(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/73478 A2

(51) International Patent Classification[7]: C12N 15/861, 15/864, 15/10, A61K 48/00

(21) International Application Number: PCT/US00/15442

(22) International Filing Date: 1 June 2000 (01.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/137,213 1 June 1999 (01.06.1999) US
60/161,097 22 October 1999 (22.10.1999) US

(71) Applicant (*for all designated States except US*): UNIVERSITY OF WASHINGTON [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105-4631 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): LIEBER, André [DE/US]; 7251 29th Avenue, Seattle, WA 98115 (US). SHAYAKHMETOV, Dmitry [RU/US]; 6500 25th Avenue, Seattle, WA 98115 (US). FARRAR, Denise [US/US]; 1324 W. Emerson Street, Seattle, WA 98119 (US). PAPAYANNOPOULOU, Thalia [US/US]; 702 35th Avenue, Seattle, WA 98122 (US).

(74) Agent: ADRIANO, Sarah, B.; Mandel & Adriano, Suite 60, 35 No. Arroyo Parkway, Pasadena, CA 91103 (US).

(81) Designated States (*national*): AE, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/73478 A2

(54) Title: RECOMBINANT ADENOVIRAL VECTORS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION AND EXPRESSING CHIMERIC FIBER PROTEINS

(57) Abstract: The present invention provides for novel chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_v\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

RECOMBINANT ADENOVIRAL VECTORS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION AND EXPRESSING CHIMERIC FIBER PROTEINS

This invention was made, at least in part, with funding from the National Institutes of Health (Grant Nos. R01 CA 80192-01 and R21 DK 55590-01). Accordingly, the United States Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the field of gene therapy, and in particular, to novel adenovirus (Ad) vectors that selectively infect cells for gene therapy, and to Ad vectors containing modifications of the fiber protein to allow retargeting of any adenovirus serotype.

BACKGROUND OF THE INVENTION

Gene transfer vectors require the efficient transduction of target cells, stable association with the host genome, and adequate transgene expression in the appropriate target cell, without associated toxic or immunological side effects,. Currently available viral vector systems, including recombinant retroviruses, adenoviruses and adeno-associated viruses, are not suitable for efficient gene transfer into many cell types. Retroviral vectors require cell division for stable integration. Recombinant adenoviruses are not able to infect many cell types important for gene therapy, including hematopoietic stem cells, monocytes, T- and B-lymphocytes. Moreover, recombinant adeno-associated vectors (AAV) integrate with a low frequency.

First generation adenoviruses have a number of properties that make them an attractive vehicle for gene transfer (Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205). These include the ability to produce purified virus at high titers in concert with highly efficient gene transfer of up to 8 kb long expression cassettes into a large variety of cell

types *in vivo,* including non-dividing cells. Limitations of first generation adenoviruses include the development of immune responses to expressed viral proteins resulting in toxicity and virus clearance. The episomal status of adenoviral DNA within transduced cells is another limitation of first generation Ad vectors. Stable integration of adenovirus DNA into the host genome is reported only for wild-type forms of specific subtypes and appears not to occur in a detectable manner with E1/E3-deleted Ad 5 (adenovirus serotype 5) vectors widely used for gene transfer *in vitro* and *in vivo* [Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205].

Recombinant AAV vectors (rAAV) integrate with a low frequency (about 1 out of 20,000 genomes) randomly as cocatemers into the host genome (Rutledge, E.A.; Russel, D.W. 1997 *J. Virology,* 71, 8429-8436). The presence of two AAV inverted terminal repeats (ITRs) and as yet unknown host cellular factors seem to be the only requirement for vector integration (Xiao, X., et al, 1997, *J. Virology,* 71, 941-948; Balague, C., et al. 1997, *J. Virology*, 71, 3299-3306; Yang, C.C. 1997, *J. Virology*, 71, 9231-9247). In the presence of the large AAV Rep proteins, AAV integrates preferentially into a specific site at human chromosome 19, called AAVS1 (Berns, K.I., 1996, *Fields Virology*, Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2173-2220). The AAV capsid is formed by three coat proteins (VP1-3), which interact with specific heparin sulfates on the cell surface and probably with specific receptor(s). However, many cell types, including hematopoietic stem cells, lack these structures so that rAAV vectors based on AAV2 cannot infect or transduce these cells (Malik P. et al., 1997, *J. Virology*, 71, 1776-1783; Quing, K.Y., et al. 1998, *J. Virology*, 72, 1593-1599). Other disadvantages of rAAV vectors include the limited insert size (4.5-5kb) that can be accommodated in rAAV vectors lacking all viral genes and low transducing titers of rAAV preparations.

Adenovirus infection is initiated by attaching to the cell surface of Ad 5 via its fiber protein (for a review, see Shenk, T. 1996 *Fields Virology, Vol. 2*, Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2111-2148). The distal, C-terminal domain of the trimeric fiber molecule terminates in a knob, which binds to a specific cellular

receptor identified recently as the coxackie-adenovirus receptor (CAR) (Bergelson, J.M. et al. *Science*, 275, 1320-1323). After binding, in an event independent of virus attachment, Arg-Gly-Asp (RGD) motifs in the penton base interact with cellular integrins of the $\alpha 3$ and $\beta 5$ types. This interaction triggers cellular internalization whereby the virion achieves localization within the endosome. The endosomal membrane is lysed in a process mediated by the penton base, releasing the contents of the endosome to the cytoplasm. During these processes, the virion is gradually uncoated and the adenoviral DNA is transported to the nucleus where replication takes place. The terminal protein, which is covalently attached to the viral genome and the core protein V that is localized on the surface of the cores have nuclear localization signals (NLSs) (van der Vliet, B. 1995, *The Molecular Repertoir of Adenoviruses, Vol. 2*, Doerfler, W. and Boehm, P.(ed.), Springer Verlag, Berlin, 1-31). These NLSs play a crucial role in directing the adenoviral genome to the nucleus and probably represent the structural elements which allow adenovirus to transduce non-dividing cells. When the double-stranded, linear DNA reaches the nucleus, it binds to the nuclear matrix through its terminal protein.

Since the cell types that can be infected with Ad5 or Ad2 vectors are restricted by the presence of CAR and specific integrins, attempts have been made to widen the tropism of Ad vectors. Genetic modification of adenovirus coat proteins to target novel cell surface receptors have been reported for the fiber (Krasnykh, V. et al. 1998 *J. Virology*, 72, 1844-1852, Krasnykh, V. et al. 1996 *J. Virology*, 70, 6839-6846, Stevenson, S.D., et al. 1997, *J. Virology*, 71, 4782-4790), penton base (Wickham, T.J., et al. 1996, *J. Virology*, 70, 6831-6838; Wickham, T.J., et al. 1995, *Gene Therapy*, 69, 750-756), and hexon proteins (Crompton, J., et al. 1994, *J. Gen. Virol.* 75, 133-139). The most promising modification seems to be the functional modification of the fiber protein or more specifically of the fiber knob as the moiety, which mediates the primary attachment. Two groups have reported the generation of fibers consisting of the Ad5 tail/shaft and the knob domain of Ad3 (Krasnykh, V. et al. 1996 *supra*, Stevenson, S.D., et al. 1997, *supra*). Recently, recombinant adenoviruses with fibers containing C-terminal poly-lysine, gastrin-releasing peptide, somatostatin, E-selectin-binding peptide, or oligo-Histidines were produced in order to change the native tropism of Ad5. Krasnikh et al. found (Krasnykh, V. et al.

1998 *supra*) that heterologous peptide ligands could be inserted into the H1 loop of the fiber knob domain without affecting the biological function of the fiber. Based on studies with other Ad serotypes, it appears that the length of the fiber shaft is a critical element, determining the efficiency of interaction with cell surface integrins and the internalization process. Thus far, there is no reported data demonstrating successful retargeting of Ad5 vectors for a specific cell type.

Therefore, there is a present need for an improved adenovirus vector which can be targeted efficiently to a variety of cell types and tissues and remain stably integrated in the host genome with minimal antigenicity to the host. The present invention discloses novel chimeric adenoviral (Ad) Ad-AAV vectors, which express a modified fiber protein on their capsid, for specifically targeting the vector. Methods of making, uses and advantages of these vectors are described. In addition, the alteration described for the knob and shaft domains of the fiber protein provide a novel approach to retarget any adenovirus serotype for cell specific infection.

SUMMARY OF THE INVENTION

The present invention provides for novel chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_{\upsilon}\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

The recombinant adenovirus vectors of the invention (Example I) provide a novel design that allows for the easy production and delivery of a "gutless" adenoviral vector with the added advantage of stable integration of the transgene into the host genome of different cell type. The adenoviral vector described is devoid of all adenoviral sequences except for the 5' and 3' cis elements necessary for replication and virion encapsidation. The adenovirus-associated virus sequences of the invention comprising the 5' (right) and 3' (left) inverted terminal repeats (ITRs) flank the transgene gene cassette such that they

direct homologous recombination during viral replication and viral integration into the host genome. In one embodiment AAV-ITR flanking sequences are used. The vector also contains a selected transgene(s) operably linked to a selected regulatory element and a polydenylation stop signal, which is in turn flanked by the flanking sequences described above. The selected transgene(s)can be linked under the same regulatory elements or under separate regulatory elements in the same orientation or in opposite orientations with respect to each other. The selected transgene(s) are any gene or genes which are expressed in a host cell or tissue for therapeutic, reporter or selection purposes. This vector is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host genome. Also provided is a method to improve the integration frequency and site specific integration by incorporating an AAV rep protein into the recombinant hybrid vector.

The invention also provides chimeric fiber proteins (Example II), which includes naturally occurring fiber proteins in which a portion or portions of the sequence are modified to alter cell or tissue specificity of infection. Altered fiber protein sequences can include fiber protein domains (the knob domain, the shaft domain, and the tail domain) from other or the same adenovirus serotypes or from randomly selected peptides. A chimeric fiber protein can be entirely composed of non-naturally occurring sequences. The invention further relates to nucleic acid sequences encoding the chimeric fiber proteins. These nucleic acid sequences can be naturally occurring, a mixture of naturally occurring and non-naturally occurring sequences, or entirely non-naturally occurring sequences.

The heterologous fiber protein sequences described herein can be inserted into any adenovirus based vector which contains a capsid, rendering the virus capable of specifically infecting a given cell or tissue. Adenoviral vectors having such a heterologous fiber sequence can be used to direct gene transfer into desired cells. For stable integration of the transgene cassette into the host gemone, the chimeric Ad-AAV vector described in the invention is the preferred vector of use.

The invention also includes a library of adenoviruses displaying random peptides in their fiber knobs can be used as ligands to screen for an adenovirus variant with tropism to a particular cell type *in vitro* and *in vivo*.

The chimeric Ad- vectors described herein include the Ad.AAV genome with a modified fiber protein expressed on its capsid. These chimeric vectors are designed to infect a wide variety of cells, in particular, the cells which can only be poorly transduced by the commonly used retroviral, AAV and adenoviral vectors. These cells include, but not limited to, hematopoietic stem cells, lung epithelial cells, dendritic cells, lymphoblastoid cells, and endothelial cells. Hematopoietic stem cells such as CD34+ cells can be targeted for gene therapy of sickle cell anemia and thalasemia using the vector described herein. The chimeric Ad-AAV vector capable of transducing genes into endothelial cells can be used in gene therapy for vascular diseases such as atherosclerosis or restinosis after coronary artery surgery.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1C display a proposed mechanism for forming of ΔAd.AAV1 genome.

Figs. 2A and 2B show electron photomicrographs of hybrid virus particles: Fig. 2A shows Ad.AAV1 and Fig. 2B shows ΔAd.AAV1.

Fig. 3 illustrates analysis of ΔAd.AAV1 genomes after transduction of SKHep1 cells. Pulse field gel eletrophoresis (PFGE). 1×10^6 control Sk Hep1 cells (SKHep1) (lanes 1-3, 5, 9). SKHep1 cells from G418 resistant pools (ΔAd.AAV1) (infected with ΔAd.AAV1 and selected for 4 weeks) (lanes 6-8, 10-12), or SKHep1 cells collected at 3 days after infection with 2000 genomes Ad.AAV1 (Ad) lanes 4, 13) are sealed in agarose plaques, lysed *in situ* and subjected to PFGE with or without prior digestion with restriction endonucleases. Southern Blot is performed with a SEAP specific probe. U = undigested, P = digested with PI- Sce1, I = I-CeuI, E = EcoREI.

Figs. 4A and 4B show response of K562 and CD34+ cells respectively after infection with ΔAd.AAVBG. Cells are incubated for 6 hours with virus under agitation. At day 3 after infection, transduction frequency is calculated based on the number of X-Gal positive cells. Viability is tested by trypan blue exclusion. N = 3, SEM < 10%.

Fig. 5 shows Rep expression in SKHep1 and 293 cells after plasmid transfection. $5x10^5$ cells are transfected with pAAV/Ad, pRSVrep, or pPGKrep by Ca-phosphate co-precipitation. Three days after transfection, cells are harvested. Lysates are separated on a 10% PA gel, followed by Western Blot with Rep specific antibodies (03-65169), American Research Products), and developed with ECL (Amersham).

Fig. 6 shows detection of vector integration into AAVS1 by PFGE.

Fig. 7 shows strategy for creating an ΔAd.AAV hybrid vector capable of site-specific integration. Arrows indicate promoters, (PA) = polyadenylation signal. Ψ = adenoviral packaging signal.

Fig. 8A-8B shows vectors for transduction studies with SNori as expression unit and analysis of vector integration on genomic DNA from a small cell number. Analgous vector sets can be generated with β-galactosidase (BG) or green fluorescence protein (GFP) as reporter genes.

Fig. 9 shows strategy for substituting the Ad5 fiber sequence by the heterologous fiber X genes using recombination in *E. coli.*

Fig. 10 shows the expression of CAR and α_v-integrins on test cells. For flow cytometry analysis, HeLa, CHO, K562, and CD34+ cells were incubated with monoclonal anti-CAR (RmcB, 1:400 dilution) or anti-α_v-integrin antibodies (L230, 1:30 dilution). As a negative control, cells were incubated with an irrelevant mouse monoclonal antibody (anti-BrdU, 1:100 dilution). The binding of primary antibodies was developed with anti-mouse IgG-

FITC labeled conjugates (1:100 dilution). Data shown represent the average results of quadruplicate analyses performed on 10^4 cells.

Fig. 11 shows the electron microscopy of adenovirus particles. Purified particles from Ad5, 9, and 35 were negative contrast stained and analyzed at a magnification of 85,000x. Defective particles are highlighted by arrows.

Fig. 12 shows the analysis of attachment and internalization of different serotypes to CHO, HeLa, K562, and CD34+ cells. Equal amounts of [^{3}H]-thymidine-labeled virions of Ads 3, 4, 5, 9, 35, and 41 (measured by OD_{260}, and equivalent to an MOI of 400 pfu per cell for Ad5) were incubated for one hour on ice as described in Materials and Methods. Cells were then washed, and the number of labeled virions bound per cell was determined. For internalization studies, viruses were first allowed to attach to cells for 1 h on ice. Then, unbound viral particles were washed out. Cells were then incubated at 37°C for 30 min followed by treatment with trypsin-EDTA and washing to remove uninternalized viral particles. The data were obtained from two to four independent experiments performed in triplicate. Note the different scale on the Y-axes for CD34+ cells.

Figs. 13A-13C show attachment and internalization of different adenovirus serotypes to Hela, CHO and 293 cells respectively.

Figs. 14A and 14B show attachment and internalization of different adenovirus serotypes to CD34+ and K-562 cells respectively.

Fig. 15A-15C shows the analysis of viral replication in K562 and CD34+ cells by Southern blot analysis of methylated viral DNA. Replication studies were performed with 1×10^5 K562 cells (A) or CD34+ cells (B), infected with methylated Ad5, Ad9 or Ad35. The lane labeled as "load" represents DNA that was extracted form the media/cell mixture immediately after adding the indicated viral dose to cells. The intensities of bands corresponding to methylated and un-methylated viral DNA indicate that ~85% of

the input virus was methylated. To quantify adsorption and internalization, DNA analysis was performed after prior incubation of virus with cells at 0^0C (adsorption) or 37^0C (internalization). For dose dependent replication studies, the indicated viral dose (expressed as the number of genomes) was added to the cells, and cellular genomic DNA together with viral DNA was extracted 16 hours or 36 hours post-infection for K562 and CD34+cells, respectively. Identical amounts of sample DNA were analyzed by Southern blot. For quantification purposes, Ad9 replication was analyzed together with Ad5 using an Ad5/9 chimeric probe that hybridizes with DNA of both serotypes (C). The analysis of Ad5 versus Ad35 replication was performed with the corresponding Ad5/35 chimeric probe. Since separate hybridizations with both Ad5/35 and Ad5/9 probes gave identical signal intensities for Ad5 DNA only one panel is shown for Ad5 replication in test cells. To produce distinguishable fragments specific for the methylated or non-methylated status of viral genomes, Ad5 DNA was digested with *Xho* I, while Ad9 and Ad35 DNA was digested with *Xho* I and *Hin*d III. The bands specific for methylated (not-replicated) viral DNA were ~12kb for Ad9, 35kb for Ad5, and ~12kb for Ad35. The fragments specific for non-methylated DNA were 5.8kb for Ad9, 6.1kb for Ad5, and 9.5kb for Ad35. Chimeric Ad5/9 and Ad5/35 DNA fragments (1.8kb) were used as quantification standards and applied onto gel together with digested viral/cellular DNA (shown on the left part of the figures).

Fig. 16A-16B shows the structure of Ad5GFP and chimeric Ad5GFP/F35 vectors. A) Schematic diagram of the original E1/E3 deleted Ad5-based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.coli. *Kpn* I (K) and *Hin*d III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes. A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The

Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal. The region of Ad5GFP/F35 encoding for chimeric fiber was completely sequenced with Ad5 specific primers (see Material and Methods). B) Restriction analysis of viral genomes. Viral DNA was isolated from purified Ad5GFP and Ad5GFP/F35 particles as described elsewhere. One microgram of DNA was digested with *Hin*d III or *Kpn* I and separated in ethidium bromide stained agarose gels (left panel) which were subsequently blotted and analyzed by Southern blot with an Ad5 E4 specific probe (nt 32,7775-33,651) (right panel). Specific patterns, designating the correct structure for both viral vectors were detected. The *Hin*d III fragments specific for Ad5GFP and Ad5GFP/F35 were 2.9kb and 4.9kb, respectively. The *Kpn* I fragment that confirmed the correct Ad5GFP/F35 structure was 1.6kb compared to a 7.6kb Ad5GFP fragment. M - 1kb ladder (Gibco-BRL, Grand Island, NY).

Fig. 17 shows the generation of ΔAd.AAV genenomes by recombination between inverted homology regions. Recombination between two inverted repeats (IR) present in one Ad.AAV vector. The first-generation Ad.AAV vector (~34kb) contains two 1.2kb inverted homology elements flanking the transgene cassette. One AAV-ITR is inserted between the Ad packaging signal (ψ) and the left IR. During Ad replication, recombination between the Irs mediates the formation of the ΔAd.AAV genomes with the transgene flanked by Irs, AAVITRs, Ad packaging signals, and Ad ITRs. These genomes are efficiently packaged into Ad capsids.

Fig. 18 shows the structure of Ad5/11, Ad5/35. Schematic diagram of the original E1/E3 deleted Ad50based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.col. Kpn I (K) and Hind III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes.

A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal.

Fig. 19 shows the cross-competition for attachment and internalization of labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled viruses, and with anti-CAR or anti-α_v-integrins Mab. (A) For attachment studies, 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled competitor virus at 4°C for 1 h. Then, equal amounts of [^{3}H]-labeled viruses, at a dose equivalent to an MOI of 100 pfu per cell determined for Ad5GFP, were added to cells followed by incubation at 4°C for 1 h. Cells were then washed with ice-cold PBS, pelleted and the percentage of attached virus (cell-associated counts per minute) was determined. For analysis of cross-competition for internalization, cells were pre-incubated with a 100-fold excess of competitor virus at 37°C for 30 min before labeled virus was added. After an additional incubation at 37°C for 30 min, cells were treated with trypsin-EDTA for 5 min at 37°C, washed with ice-cold PBS, pelleted, and the percentage of internalized virus was determined. For controls, cells were incubated with labeled viruses without any competitors. Preliminary experiments had shown that the conditions chosen for competition studies allowed for saturation in attachment/internalization on K562 cells for all unlabeled competitors. (B) 10^5 K562 cells were pre-incubated for 1 hour at 4°C with anti-CAR MAb (RmcB, diluted 1:100) or with anti-α_v-integrin MAb (L230, diluted 1:30), followed by incubation with labeled viruses according to the protocols for attachment or for internalization as described above. For each particular serotype, the percentage of attached/internalized virus was compared to the control settings, where cells were preincubated under the same conditions with a 1:100 dilution of an irrelevant antibody (anti-BrdU Mab) before addition of the labeled virus. Note that the specific competitors but not the corresponding controls significantly inhibited Ad5 internalization to a degree that is in agreement with published data (59). N>/=4

Fig. 20. Cross-competition for attachment and internalization of [^{3}H]-labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled Ad3 virus (A), and of [^{3}H]-

labeled Ad3 virions with unlabeled viruses (B). 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled viral particles according to attachment or internalization protocols described for Fig.6. Equal amounts of [^{3}H]-labeled Ad5GFP, Ad5GFP/F35, or Ad35 (A) or [^{3}H]-labeled Ad3 (B) were added to cells at a dose equivalent to an MOI of 100 pfu per cell for Ad5GFP. In control settings, cells were incubated with labeled viruses without any competitors. N=4.

Fig. 21 shows the transduction of CD34+, K562, and HeLa cells with Ad5GFP and chimeric Ad5GFP/F35 vectors. $1x10^5$ cells were infected with different MOIs (pfu/cell) of viruses in 100 μl of media for 6 hours at 37°C. Virus containing media was then removed, and the cells were resuspended in fresh media followed by incubation for 18 h at 37°C. The percentage of GFP expressing cells was determined by flow cytometry. N=3

Fig. 22 shows the distribution of GFP-positive cells in subpopulations of human CD34+ cells expressing CAR or α_v-integrins. $1x10^5$ CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu/cell as described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CAR (1:100 final dilution) or anti-α_v-integrin (1:30 final dilution) primary MAbs for 1 h at 37°C. Binding of primary antibodies was developed with anti-mouse IgG-PE labeled secondary MAbs (1:100 final dilution) at 4°C for 30 min. For each variant, 10^4 cells were analyzed by flow-cytometry. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The data shown were representative for three independent experiments.

Fig. 23A-23B shows the distribution of GFP-positive cells in a subpopulation of human CD34+ cells, expressing CD34 and CD117 (c-kit). (A) Co-localization of GFP expression with CD34 or CD117: CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell under the conditions described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CD34 PE-conjugated MAbs (final dilution 1:2) or with anti-CD117 PE-conjugated MAbs (final dilution 1:5)

for 30 min on ice, and 10^4 cells per variant were subjected to two-color flow cytometry analysis. For negative control staining, no antibodies were added to the cells before analysis. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The experiment was performed two times in triplicates, and typically obtained results are shown. The SEM was less than 10% of the statistical average. (B) Transduction of CD34+/CD117+ cells with Ad5GFP and chimeric Ad5GFP/F35 virus vectors: CD34+ cells, cultured overnight before staining in media without SCF, were incubated with PE-labeled anti-CD117 MAb for 30 min on ice. The fraction of CD117-positive cells was sorted by FACS. More than 97% of sorted cells were positive for CD117. $1x10^5$ CD117+/CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell, as for Fig.8. Twenty-four hours post infection, the percentage of GFP positive was determined by flow cytometry. For mock infection, CD117+/CD34+ cells were incubated with virus dilution buffer only. The infections were done in triplicates, and the average percentage of GFP-expressing cells is indicated on the corresponding histogram. The SEM was less than 10% of the statistical average.

Fig. 24 shows the southern analysis of viral genomes in GFP-positive and GFP-negative fractions of CD34+ cells infected with the Ad5GFP and chimeric Ad5GFP/F35 vectors. CD34+ cells were infected with viruses at an MOI of 100 as described for Fig.21. Twenty four-hours post infection, cells were sorted by FACS for GFP positive and GFP negative fractions. 10^5 cells from each fraction were used to isolate genomic DNA together with viral DNA. Before cell lysis, a rigorous treatment with trypsin and DNase followed by washing was performed to exclude that genomic DNA samples were contaminated by extracellular viral DNA. A) The upper panel shows the ethidium bromide stained 1% agarose gel before blotting demonstrating that similar amounts of genomic DNA were loaded. This amount corresponded to DNA isolated from ~25,000 GFP+ or GFP- cells. The lane labeled Aload@ represents viral DNA purified from Ad5GFP or Ad5GFP/F35 virions mixed with pBluescript plasmid DNA (Stratagene) as a carrier and applied on a

gel at the amount that was actually used to infect 25,000 cells. As a concentration standard, a serial dilution of Ad5GFP genomes was loaded on the gel (left side). For Southern analysis (lower panel), an 8kb-long HindIII fragment corresponding to the E2 region of Ad5 was used as a labeled probe. Hybridized filters were subjected to PhosphoImager analyis and then exposed to Kodak-X-OMAT film for 48 h at B70°C. The cellular/viral genomic DNA is indicated by an arrow. (B) To detect Ad5GFP genomes in transduced cells, PCR amplification followed by Southern blot hybridization was performed on the same samples that were used for quantitative Southern blot hybridization in (A). DNA purified from ~2,500 cells was subjected to PCR (95°CB1min, 53°C-1min, 72°CB 1min, 20 cycles with primers Ad5-F1 and Ad5-R1). One fifth of the PCR reaction was subjected to agarose gel electrophoresis (upper panel). A 0.9 kb-long DNA fragment, specific to the E4 region of Ad5 was detected for transduced Ad5GFP/F35 genomes. DNA then was blotted onto Nybond-N+ membrane and Southern blot hybridization (lower panel) with an Ad5 E4 specific DNA probe was performed. In addition to the 0.9kb DNA fragment, the PCR primers generated a smaller 0.5 kb-long fragment that also hybridized with with the E4 region probe.

Fig. 25 shows the role of fiber shaft length in Ad infection strategies. CAR binding (Ad5 and Ad9) variants and Ad35, which interacts with a non-CAR receptor were analyzed on CAR expressing cells (293, Y79) and K562 cells which do not express significant CAR amounts. All vectors contain a GFP expression cassette packaged into an Ad5 capsid with modified fibers.

Fig. 26 shows the tertiary structure of Ad5 knob: localization of CAR binding sites, H-I loop and G-H loop.

Fig. 27 shows the substitution of the G-H loop with heterologous peptides.

Fig. 28 shows the attachment and internalization of metabolically labeled serotypes with human cell lines.

Figs. 29A-29D shows the generation of Rep78 expressing Ad vectors by recombination between two vectors. (A) The same strategy outlined in Fig. 15 was employed for vectors with rep 78 as a transgene. The Ad5'rep vector also contained the ApoEhAAT promoter shielded by an HS-4 insulator. The region of homology between the two fragments of the rep78 gene was 658nt in length. The Rep78 ORF was deleted for the p5 promoter. The internal Rep 40/52 start codon (at position 993) was mutated to abolish production of the small Rep proteins. Furthermore, the splice site at nt 1905 was deleted eliminating production of Rep68. The individual expression of Rep 78 was demonstrated. (B) Formation of ΔAd.rep78 genomes. The expected 5.8kb ΔAdrep78 genome was only observed upon coninfection of both Ad5'rep and Ad3'rep into 293 celss as demonstrated by Southern. (C) Southern blot analysis for rescue of the recombinant AAV genome from plasmid DNA by Rep78 expressed from pCMVrep78 and ΔAd.rep78. The expected rescue product is 3.8kb (R-plasmid). (D) Southern blot analysis for rescue of the recombinant AAV genome from Ad.AAV viral vector genomes.

DETAILED DESCRIPTION OF THE INVENTION

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in in the present invention, the following words or phrases have the meanings specified.

The term vector includes, but is not limited to, plasmids, cosmids, phagemids, and artificial chromosomes. The vector sequence may be designated as the viral "base vector" sequence. The base vector sequence is dependent upon the particular type of virus and serotype that the base vector sequence was derived from. The base vector sequence may be linked to non-vector or transgene sequences (e.g., heterologous sequences).

The transgene sequences may include sequences that confer compatibility with prokaryote or eukaryote host cells, where compatibility relates to vector replication

within a host cell. Accordingly, the transgene sequence may be a replicon sequence that directs replication of the vector within the host cell, resulting in an autonomously replicating vector. Alternatively, the transgene sequence may permit vector replication that is dependent upon the host cell's replication machinery.

The base vector sequences may be a operatively linked to a transgene sequence that encodes a gene product, such as a polypeptide, rRNA, or tRNA. For example, the transgene may encode a polypeptide such as a viral capsid protein, or a viral fiber protein. The transgene may be derived from the same or different serotype as the base vector sequence.

Another example of a transgene includes a reporter gene that encodes a gene product that can be used as a selectable marker, such as drug resistance or a colorimetric marker. The reporter gene may encode a gene product which can be readily detected by, for example, a visual microscopic, immunochemical, or enzymatic assay. The preferred reporter gene encodes a gene product that can be detected by a non-destructive method that does not destroy the cell that expresses the reporter gene.

A therapeutic gene is another example of a transgene. A therapeutic gene encodes a gene product (e.g., polypeptide or RNA) which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or the tissue or the organ or the organism containing the host cell. The therapeutic benefit may result from modifying a functin of a gene in the host genome or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The base vector sequence may be linked to a transgene sequence that is an regulatory element, such as a promoters, enhancers, transcription termination signals, polyadenylation sequences. The regulatory element may direct expression of the transgene sequence that encodes a gene product by direct transcription or translation. The regulatory element may regulate the amount or timing of expression of the transgene

sequence. The regulatory element may direct expression of the transgene in certain host cells or tissues (e.g., host-specific or tissue-specific expression).

The base vector sequence may linked to a transgene sequence that permits the vector, to integrate into another nucleotide sequence. The integration sequence may direct integration of the whole vector or portions of the vector. The integration sequence may or may not be related to the base vector sequence. For example the integration and base vector sequences may be from the same or different viral serotype. The integration sequence may be inverted repeat sequences (ITRs) from adenovirus (Ad), adenovirus-associated virus (AAV), or HIV.

The base vector sequence may be linked to a transgene sequence that directs homologous recombination of the vector into the genome of a host cell. Such transgene sequences may or may not be from the same viral serotype as the base vector sequence.

The vector may be used to transport the heterologous sequence into a host cell or into a host cell's genome.

The vector may comprise multiple endonuclease restriction sites that enable convenient insertion of exogenous DNA sequences.

The term "hybrid vector" as used in the invention refers to a vector which comprises a nucleic acid sequence combined from two different viruses (e.g. Adenovirus and AAV).

"Chimeric vector" refers to a vector which contains nucleic acid sequences that are unnatural to the base vector (i.e. sequences not occurring naturally or sequences not in their natural background including heterologous sequences). A chimeric vector as used in the invention may also be a hybrid vector. An example of a chimeric vector is Ad.AAV expressing a modified fiber protein an its capsid.

The term "transduction" or "infection" refers to a method of introducing viral DNA within a virus particle into a host cell. The viral DNA herein is in the form of recombinant virus, which is generated by linking a segment of DNA of interest into the viral genome in such a way that the gene can be expressed as a functional protein.

The term "transfection" refers to a method of introducing a DNA fragment into a host cell.

The term "heterologous" as used herein means that a nucleic acid or peptide sequence is placed in a context that is not endogenous to the base adenovirus vector or to a transduced cell. For example, a peptide sequence can be transferred from a protein to another protein, the resulting protein is referred to herein as heterologous protein. A chimeric fiber protein, (e.g., a serotype 5 tail domain and a serotype 35 shaft and knob domain) is considered a "heterologous" to the Ad 5 vector. The term also includes nucleic acids (e.g. coding sequences) from one strain or serotype of adenovirus introduced into a different strain or serotype of adenovirus.

The term "regulatory elements" is intended to include promoters, enhancers, transcription termination signals, polyadenylation sequences, and other expression control sequences. Regulatory elements referred to in the invention include but are not limited to, those which direct expression of nucleic acid sequence only in certain host cells (e.g. tissue specific regulatory sequences).

The term "operably linked" indicates that a polynucleotide sequence (e.g. a coding sequence or gene) is linked to a regulatory element in such a way that the regulatory element sequence controls and regulates the transcription or translation or both of that polynucleotide sequence. The orientation of the regulatory element may vary (eg, be in reverse orientation with respect to the right ITR). The term also includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence and

production of the desired polypeptide or protein. Regulatory sequences can also include 3' sequences which ensure correct termination (eg. polyadenylation stop signal).

The term "gene therapy" used herein, refers to a method which introduces a segment of exogenous nucleic acid into a cell in such a way that it results in functional modification to the recipient cell by expression of the exogenous nucleic acid. The exogenous nucleic acid is typically therapeutic in that the expression of the encoded protein, polypeptide or RNA corrects cellular dysfunction due to a genetic error or more generally counteracts any undesirable functions which are associated with a genetic or acquired disease. The term "exogenous nucleic acid" refers to DNA or RNA sequences not normally expressed in the treated transformed cell. The term also refers to DNA and RNA sequences which are expressed in a treated transformed cell at a higher, lower or in an otherwise different pattern than in the untreated, nontransformed cell. This non-natural expression can also be termed heterologous expression.

A "gene therapy vector" refers to a vector used for gene therapy. i.e. to introduce the exogenous nucleic acid into a recipient or host cell. The exogenous nucleic acid may be transiently expressed or integrated and stably expressed in the recipient or host cell.

The term "plasmid" as used herein refers to any nucleic acid molecule which replicates independently of the host, maintains a high copy number, and which can be used as a cloning tool.

The term "parallel strand of DNA" and "anti-parallel strand of DNA" refers to as each of the strands of DNA of the double stranded adenovirus. The Figures diagram the location of certain nucleotides on the parallel strand of DNA. The anti-parallel strand of DNA refers to the other of the two strands of DNA which is not depicted in the Figures. The fiber protein is encoded on the anti-parallel strand of DNA. To simplify the vector diagrams, the fiber sequences are shown on the parallel strand even though the gene is located on the anti-parallel strand.

The term "reporter gene" refers to any nucleic acid sequence which encodes a polypeptide or protein which can be readily detected by, for example, a visual, microscopic, immunochemical or enzymatic assay. Preferred reporter genes are those that can be detected by a non-destructive method that does not destroy the treated, transformed cells or tissue.

The term "selection gene" used herein refers to any nucleic acid fragment which encodes a polypeptide or protein whose expression is used to mark a cell as a transformed cell by a given vector.

The term "therapeutic gene" refers herein to a DNA fragment encoding a functional polypeptide, protein or RNA, which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or to the organ or organism containing the host cell. The therapeutic benefit may result from modification of a function of a native gene in a host or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The term "host tissue" or "host cell" as used herein, refers to a tissue or cell in which a therapeutic gene is to be expressed to modify its function.

It is well-known in the biological arts that certain amino acid substitutions may be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate, and isoleucine and valine, are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Volume 5, Supplement 3, Chapter 22, pp. 345-352, which is incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff et

al's frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources. Therefore, any obvious changes in the amino acid sequences (as described above) to the sequences of the invention are already contemplated.

Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups include but are not limited to: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, asparagine-glutamine, and aspartate-glutamate. Therefore, polypeptide substitution for "substantially similar" sequences (as described above) to the amino acid sequences described invention are already contemplated.

In order that the invention herein described may be more fully understood, the following description is set forth.

The present invention provides unique gene transfer vehicles which overcome many of the limitations of prior art vectors. The invention describes a first generation adenovirus vectors comprising left and right Ad ITRs, an Ad packaging sequence, a transgene cassette with regulatory elements, and a pair of cassette ITRs flanking the transgene cassette that direct predictable viral genomic rearrangements during viral replication as well as direct the integration of the transgene cassette into the host cell genome.

One predictable rearrangement that occurs during viral replication is the generation of a gutless adenovirus vector (also referred to herein as ΔAd) that comprises right and left Ad ITRs, an Ad packaging sequence, a transgene cassette flanked by cassette ITRs and the gutless vector is devoid of all other immunogenic viral genes.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the transgene cassette is directed by co-infection with an Ad vector expressing e.g., the rep 78 protein to achieve site-specific integration in the e.g., AAVS1 site on human chromosome 19.

The invention further describes a novel way of targeting these recombinant adenovirus vectors to selected cells by modifying the adenovirus fiber protein that is expressed on the capsid. Changes to both the fiber shaft and the fiber knob domain proved to successfully retarget the Ad vector to a desired cell type. In addition, the G-H loop within the fiber knob domain is identified as a novel site that affect the binding affinity and specificity of the recombinant adenovirus vector. Substitution of peptide sequences into the G-H loop retarget the gutless vector to a desired cell type.

An adenovirus display library has been generated that expresses random peptides within the G-H loop of the fiber protein. This type of a library is used as ligands to screen for adenovirus vectors that bind to desired cell types. One advantage of using an adenovirus display library versus a phage display library is that once adenovirus affinity to a desired cell is identified the targeted adenovirus vector is ready to accept a transgene cassette and can be used to generate a gutless adenovirus vector, for example for use in gene therapy.

The chimeric Ad vectors described below contain a modified fiber protein in the capsid of the adenovirus which renders the vector capable of infecting a desired cell types. Therefore, according to the invention, a gutless chimeric ΔAd-AAV vector can be generated to introduce any transgene(s) into any host cell or tissue which is normally refractory to most commonly used gene therapy viral vectors. In addition, the chimeric ΔAd.AAV vector of the invention, is devoid of adenoviral genes, and contains AAV ITR

sequences that flank the transgene cassette, which direct stable transgene integration in the host genome allowing long term expression of the transgene.

The transgene cassette described in the invention may carry a transgene which is either a reporter gene, a selectable gene for in vitro or in vivo selection of transduced cells, or a therapeutic gene. In one embodiment of the invention the reporter trangene can be but is not limited to, βgalactosidase. Many reporter genes are commonly used in the art, of which any could be carried as a transgene in the Ad.AAV vector of the invention. Other examples of reporter are genes are GFP and alkaline phosphatase.

The following describes an embodiment of the first generation Ad vectors of the invention having a wild-type capsid and a transgene cassette flanked by cassette ITR sequences; (b) fiber protein that is modified to retarget Ad vectors; and (c) the combination of both technologies that enables the production of chimeric ΔAd vector including a modified fiber protein expressed on the capsid which retargets the base vector to a desired cell type for infection and transgene integration.

A. Integrating Ad hybrid vectors of the invention:

It has been shown that inverted repeats (IRs) inserted into the E1 region of AdE1- vectors can mediate predictable genomic rearrangements resulting in a gutless vector genome devoid of all viral genes. A specific embodiment of such IR-mediated rearrangements is the Adeno-AAV, first generation hybrid adenovirus vector containing AAV inverted terminal repeats (ITR) flanking a transgene cassette. The AAV ITRs mediate the formation of a genome similar to that of the ΔAd.IR genome (Steinwaerder et al., 2000 Journal of Virology). ΔAd vectors devoid of all viral genes stably integrate and transduce cultured cells with efficiencies comparable to e.g. rAAV vectors. The Examples demonstrate by Southern blot analysis that the ΔAd vectors integrate randomly into the host genome.

The Ad vectors of the invention comprise a left Ad ITR, an adenovirus packaging sequence located 3' to the Ad ITR; a transgene cassette located 3' to the packaging

sequence comprising a polyadenylation signal, a transgene, and a heterologous promoter, and flanked by a pair of cassette ITRs. Adenoviral genes used for replication such as E1, E2, E3, E4 are located 3' to the right cassette ITR and a right Ad ITR is located 3' to the replication genes. The vectors of the invention are particularly suited to treat: genetic disorders, cancers, and infectious diseases (such as HIV, emboli, or malaria). Treatable genetic diseases such as hemophilia A and B; cystic fibrosis; muscular dystrophy, and $\alpha_1$antitrypsin disorder are ideal candidates for genetic disease that can be treated by vectors of the invention. A specific example of a therapeutic gene to combat a genetic disorder is gamma-globin to ameliorate sickle cell anemia.

To aid in the selection of transduced cellls and characterize the intergration site of the transgene cassette, an embodiment of the invention includes the addition of a sequence comprising a bacterial for the origin of replication, plus a selectable gene. An embodiment of this is an SNori sequence added to the transgene cassette. This allows the ΔAd to be expressed in human and bacterial cells, therefore allowing selection of the transduced cells and characterization of the integration site in the genome of transduced mammalian cells.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein in 293 cells to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

Integration of the transgene cassette contained in the adenoviral vectors into chromosomes may be associated with silencing (or blocking) of transgene expression. The silencing of transgenes can be overcome by adding insulator elements to the transgene cassette. For example, HS-4 insulator elements derived from the chicken-globin LCR can function in Ad vectors to shield heterologous promoters from adenoviral enhancers. HS-4 insulators or the *Drosophila* Gypsy gene can also be used to prevent silencing transgenes.

Another embodiment of the invention is to split the transgene cassette into two portions of the transgene each carried in a different recombinant adenoviral vector of the invention. Each portion of the same trangene has an overlapping region of homology. After infection with both vectors, each carrying the different but overlapping portion of the same transgene, homologous recombination event occurs resulting in the reconstitution of the complete transgene which is then expressed. This technique is used to produce hybrid adenoviral vectors that accommodate large inserts including, but not limited to a 13kb genomic hAAT gene or a 12kb $\tilde{\gamma}$globin LCR$\tilde{\gamma}$globin expression cassettes for ameliorating sickle cell anemia (or correcting γ-globin mutations). The formation of the hybrid ΔAd vector genomes, after recombination between two vectors, is more efficient if the overlapping region of homology within the transgene is longer.

An advantage of the present invention is a method to rapidly isolate pure gutless hybrid adenoviral vectors such as ΔAd.AAV or ΔAd.AAV^{fx} vectors. To minimize the contamination of ΔAd with first generation vectors (Ad vectors) a strategy is described in Example I H. It is anticipated that these approaches will yield the same titer of ΔAd vectors, however the contamination with full-length genome vectors will be less. This improved isolation of the vectors is extremely important to avoid toxic side effects after in vivo application.

B. <u>Tropism modified adenovirus vectors:</u>

The Ad vectors of the invention can be modified so that they target a host cell of interest. There are more than 50 human Ad serotypes (Appendix I), including variants with different tissue selectivity or tropism. It is accepted in the art that different Ad serotypes bind to different cellular receptors and use different entry mechanisms. Most recombinant adenovirus vectors use adenovirus serotype 5 as the base vector serotype 5 (Ad5) (Hitt, M.M., et all, 1997, Adv. in Pharmacology 40, 137-205). Ad5 infection is primarily mediated by its fiber protein binding to CAR and secondarily by its penton base protein binding to integrin. Due to the lack of CAR and/or integrin expression on many cell and tissue types, Ad5 mediated gene transfer is inefficient in a number of tissues which are important targets for gene therapy such as endothelia, smooth muscle, skin epithelia, differentiated airway epithelia, brain tissue, peripheral blood cells, or bone marrow. The following describes Ad5 vectors of the invention having a change in infectivity and tropism as a result of altering the fiber protein sequence.

The infectivity of different Ad serotypes is limited to a number of human cell lines. Infectivity studies revealed that Ad5 and Ad3 are particularly suitable for infecting and targeting endothelial or lymphoid cells, whereas Ad9, Ad11 and Ad35 efficiently infected human bone marrow cells. Therefore, the knob domain of the fiber protein of Ad9, Ad11 and Ad35 are excellent candidates for retargeting the Ad5 vector to human bone marrow cells. Other possible serotypes include Ad7.

In the modified fiber protein of the invention the fiber knob domain of the Ad5 fiber has been replaced with another Ad serotype fiber knob domain. An embodiment of the invention is the modified Ad5/35 fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising of a fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad35). The Ad5/35 chimeric fiber protein shows a broader spectrum of infection to a subset of CD34+ cells, including those with stem cell activity. The Ad5/11 chimeric fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising the fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad11) showed similar tropism.

In addition to the knob domain modifications, the invention describes the added advantage of modifying both the fiber shaft domain and the fiber knob domain to produce a shortened fiber protein. The length of the fiber shaft domain plays a key role in the host receptors used for viral vector entry into the host cell. To show this Ad5, Ad5/9, and Ad5/35 variants were constructed with long (22β-sheets) and short-shafted (7β-sheets)-shafted fibers. These analyses demonstrated that efficient viral infection involving CAR as the primary receptor for Ad5, Ad5/9 requires a long-shafted fiber protein, whereas the cell entry strategy of Ad5/35 (which binds to an still uncharacterized non-CAR receptor) does not depend on the shaft length (Fig. 3). The modification in both the fiber shaft domain length (between 5 > 10 β-sheets) and the fiber knob domain (from a different Adserotype than the base vector is a novel mode of altering Ad vector tropism.

To broaden the repertoire of cell types that Ad vectors can infect, a specific binding region, the G-H loop, within the knob domain has been newly identified herein to improve binding affinity and specificity. Alteration within this region will redirect the Ad vector to a desired cell type. For example, the invention describes the G-H loop sequence within the fiber protein knob domain, which can be replaced with heterologous peptide ligand sequences without affecting the functionally important tertiary structure of the Ad fiber knob domain, while changing the binding affinity and specificty of the vector (Figs. 6,7). This G-H loop region is exposed on the central part of the knob surface and may be strategically a better site for incorporation of heterologous ligands than the peripheral H-I loop (Krasnykh, V. et al., 1998, *J. Virol.*, 72:1844-52.) of the knob C-terminus (Michael, S. I., et al., 1995, *Gene Ther.*, 2:660-8., Wickham, T. J. et al., 1996, *Nat. Biotechnol.*, 14:1570-3.), which are the substitution sites used by others. Therefore, these G-H loop modifications within the fiber knob domain will allow the Ad vector to be redirected to infect a desired cell type, as long as the G-H loop ligand sequence binds to at least one surface protein on the desired cell type. Fig. 7 shows some possible substitutions. Example II J demonstrates that the virion tolerates the insertion of a cycling peptide (12 amino acids) with a constrained secondary structure that allows the exposure on the knob surface. A defined ligand (RGD) can be inserted into the G-H and the H-I loop of an

Ad5 capsid that is ablated for CAR, and integrin tropism. Infectivity studies show the potential advantage of this new insertion site.

Use of the Vectors of the Invention for "Gene Therapy"

The liver is the major organ for protein synthesis. Thereforean important goal of gene therapy is to target gene therapy vectors to the liver. To genetically correct many types of mutant proteins hepatocytes need to be infected with gene therapy vectors carrying a corrected transgene. Example II J describes a G-H loop substitution in the knob domain of the fiber protein with both RI and RII+ (of the malaria circumsporozite surface protein) in a short shafted fiber protein which directs the vector to have affinity and specificity to hepatocytes.

Example II K applies a similar protocol to alter the fiber knob domain in the G-H loop region with peptides that target the vector breast cancer cell lines (MDA-MB-435). These novel approaches to redirect vectors described in the invention allow lower doses of the gene therapy vectors to be administered with a higher safety profile.

In example II L a protocol for preparing an adenovirus display library is described that uses the fiber knob protein to display a library of random peptide sequences within the G-H loop. This library of adenoviruses with modified fiber proteins is screened for affinity and specificity for a desired cell type. There are two main advantages of using this adenovirus display library to screen for target peptides that allow binding to a desired cell type over a phage display library system. First, once a ligand peptide is identified that binds to the desired cell type it is already in the vector of choice for gene therapy delivery. The peptide does not need to be engineered into another vector, as is the case for the phage display library vectors. This reduces the steps required to identify a targeted fiber protein for a desired cell type. The second advantage of this method, is that the adenoviruses are able to display multiple copies of the modified fiber protein on their capsid. This allows for dimerization and trimerization of the fiber protein with the host cell receptor. The multimerization of fibers proteins is a realistic, in vivo interaction of

the trimeric fiber protein with the host cell receptor. In contrast, phage vectors can only display one fiber peptide sequence on their surface, which significantly limits the ability of interaction with host cell surface receptors.

C. A chimeric adenovirus vector with selective tropism:

The chimeric vectors of the invention combine two vectors: an Ad.ITR and a Ad. fx where fx describes a modified fiber protein. A first generation adenovirus vector of serotype 5 is the base vector that carries a transgene cassette flanked by heterologous ITRs. These specific inverted terminal repeat sequences, such as AAV ITRs direct stable integration of the transgene cassette into the host genome as well as control predictable genomic rearrangements that occur during viral replication. This vector can also carry a modified fiber gene (described in Examples II). During replication predictable genomic rearrangements occur which result in the generation of a gutless adenovirus vector (e.g. ΔAd.AAV^{fx}) which expresses the modified fiber protein on its capsid. The modified fiber protein allows the gutless vector to be targeted to a selected cell type. The targeted vector is a gutless adenovirus vector devoid of adenoviral genes which can integrate its transgene into the host genome. The transgene cassette can carry reporter, selectable, or therapeutic genes.

In one embodiment of the invention, the gutless targeted ΔAd.AAV^{fx} carries the reporter gene of βgalactosidase. ΔAd.AAV^{fx} ¯BG). For easy in vitro selection of human and bacterial cells that are transduced with the hybrid Ad vector, a bacterial sequence for the origin of replication can be added to the hybrid Ad vectors. An example of this is ΔAd.AAV^{fx} -Snori, in which a SNori sequence is added into the transgene cassette. This site allows for G418 selection on cells infected with ΔAd.AAV^{fx} –SNori. This in vitro selection provides a tool to analyze the site of transgene integration and the flanking chromosomal regions. Fluorescent in situ hybridization (FISH) is an alternative method to confirm vector integration.

An advantage of the ΔAd chimeric vector for gene transfer is the efficient and stable integration of a large transgene cassette up to e.g. 22kb which is significantly larger than the capacity of retroviral vectors . This is of particular interest for gene therapy. For example, to ameliorate sickle cell anemia ΔAd.AAV^{fx} γglobin , an expression transgene cassette with the gamma-globin gene that targets and integrates, can be inserted into bone marrow stem cells for long term expression of the gamma-globin gene.

To achieve site-specific gene integration, rep78 protein is used for transgene integration into the AAVS1 site (described in Example 1 D). However, this may silence transgene expression. To prevent the integrated transgene from being silenced by host genomic elements (such as positional effects or downstream enhancers), LCRs or insulator elements are incorporated into the transgene cassette.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE I

NOVEL ADENOVIRAL VECTOR Ad.AAV

A. Integrating ΔAd.AAV hybrid vectors devoid of all adenoviral genes.

In vitro and *in vivo* studies with rAAV indicate that the only requirement for rAAV integration are the AAV ITRs and as yet unknown host cellular factors. It is thought that specific sequences or secondary structures present in AAV ITRs are prone to integration into host chromosomal DNA. In order to combine advantages of adenoviral vectors (high titer, high infectivity, large capacity) and the integration capability of AAV ITRs, AAV vector DNA with AAV ITRs flanking cassettes a secreted human placental alkaline phosphatase (SEAP) - neomycin phosphotransferase (neo) reporter gene cassette

(Alexander, I.E., et al. 1996, *Gene Therapy*, 7, 841-850) is incorporated into the E1-region of E1/E3 deleted adenoviral vectors (Ad.AAV1) (Figure 1, top).

METHODS

<u>*Production/Characterization of Viral Vectors*</u>

<u>*Plasmids*</u>:

The AAV1 vector cassette containing AAV ITRs and SEAP/neo expression units is obtained by AseI/ScaI digestion of the plasmid pALSAPSN (Alexander, I. E. et al. 1996, *Human Gene Therapy*. 7:841-50). The 4.4kb AAV vector fragment was cloned via NotI adapter linkers into pXJCL1 (Mirobix, Toronto, Canada) (pAd.AAV1). Another shuttle vector (pAd.AAV1-Δ2ITRs) lacking the AAV ITRs is generated by inserting the 3.7kb AflII/BsmI fragment of pALAPSN into pXJCL1. For pAd.AAV1Δ1ITR, a construct is used where a spontaneous deletion in the left AAV ITRs between the A and A' regions has occurred. To create a second hybrid vector (Ad.AAV2), the AAVSNori cassette developed by E. Rutledge is used. AAV vector DNA obtained is from pASNori (Rutledge, E. A., Russell, D.W. 1997. *Journal of Virology* 71:8429-8436) as a 3.4kb BsaI/ScaI fragment and inserted into the EcoRV site of pXCJL1. As it is generally known for AAV vector plasmids, the AAV ITRs are prone to rearrangements. To minimize deletions in these functional critical regions, all constructs for generation of hybrid vectors are assembled in low copy-number plasmids which are grown in E. coli Top10, JC811, or XL1 Bluecells (Stragene, La Jolla, Calif.). Furthermore, after each cloning step or large-scale plasmid amplification, both AAV ITRs are carefully mapped by restriction analysis with enzymes that cut inside or adjacent to the ITRs (BssHII, AhdI, SmaI, Bg1I, BsmI, AflII, and ScaI).

Adenoviruses:

First-generation viruses with the different transgene cassettes incorporated into the E1 region are generated by recombination of the pΔE1aSpla- or pXCJL1-derived shuttle plasmids and pJM17 (Microbix) in 293 cells as described earlier (Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791). For each virus, at least 20 plaques are picked, amplified, and analyzed by restriction digest. Viruses containing two AAV ITDRs tend to rearrange within the ITRs, with other adenoviral sequences, or with adenoviral sequences present in the 293 cell genome. Only plaques from viruses with intact ITRs are amplified, CsCl banded, and titered as described earlier (Kay, M. A., et al. 1995. *Hepatology* 21:815-819; Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960). All virus preparations tested are negative for RCA and bacterial endotoxin (Lieber, A., et al.1997. *Journal of Virology* 71:8798-8807). Virus is stored at –80°C in 10mM Tris-Cl, pH 7.5-1 mM $MgCl_2$-10% glycerol.

To generate ΔAd.AAV, 293 cells are infected with Ad.AAV1 at an multiplicity of infection (MOI) of 25 and harvested 40h after infection. Cells are lysed in PBS by 4 cycles of freeze/thawing. Lysates are centrifuged to remove cell debris and digested for 30 min at 37°C with 500 units/ml DNaseI and 200 μg/ml RNaseA in the presence of 10mM $MgCl_2$. 5ml of lysate is layered on a CsCl step gradient (0.5ml - 1.5g/cm^3, 2.5ml - 1.35g/cm^3, 4ml - 1.25g/cm^3) and ultracentrifuged for 2h at 35,000 rpm (rotor SW41). CsCl fractions are collected by puncturing the tube and are analyzed for viral DNA (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960; Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) or subjected to ultracentrifugation at 35,000 rpm for 18 hours in an equilibrium gradient with 1.32 g/cm^3 CsCl. The band containing the deleted viruses ΔAd.AAV is clearly separated (0.5cm distance) from other banded viral particles containing full-length ad.AAV genomes. ΔAd.AAV1 fractions are dialyzed against 10mM Tris-Cl, pH 7.5-1mM $MgCl_2$-10% glycerol and stored at –80°C. The genome titer of ΔAd.AAV1 preparations is determined based on quantitative Southern analysis of viral DNA purified from viral particles in comparison to different concentrations of a 4.4kb AseI/ScaI fragment of pALSAPSN according to a protocol described earlier (Lieber, A.,

et al., 1996, *J. of Virology*, 70, 8782-8791). In total, the production of 1×10^{13} genome-particles of ΔAd.AAV1 requires less than 3 hours of actual work.

Titers routinely obtained are in the range of $3\text{-}8\times10^{12}$ genomes per ml. Assuming one genome is packaged per capsid, the genome titer equals the particle titer. The level of contaminating Ad.AAV1 is less than 0.1% as determined by Southern analysis, which is consistent with results obtained by plaque assay on 293 cells (fewer than 5 plaques per 10^6 total genomes). The primers used for sequencing the left and right ITR-vector-junction are

5'GGCGTTACTTAAGCTAGAGCTTATCTG, and
5'CTCTCTAGTTCTAGCCTCGATCTCAC.

The recombinant AAV virus stock containing the SEAP/neo cassette (AV2/ALSAPSN, [Alexander, I. E. et al, 1996, *Human Gene Therapy,* 7:841-50] used in these studies were obtained from Dusty Miller (FHCRC, Seattle). The stock was free of contaminating replication competent AAV (<50 particles/ml) and wildtype adenovirus (<100 particles/ml). The genome titer of the virus stock was obtained by quantitative Southern Blots as described by Russell et al. (Russell, D. et al. 1994 *Proc. Natl.Acad,Sci. USA* 91:8915-8919).

Electron Microscopy:

For examination of viral particles in the transmission electron microscopy studies, CsCl-purified virions are fixed with glutaraldehyde and stained with uranyl acetate as described previously (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960).

RESULTS

During replication of these hybrid vectors in 293 cells, a 5.5kb genome (ΔAd.AAV1) is efficiently generated and packaged into adenovirus (Ad5) capsids. The ΔAd.AAV1

genome contains the left adenovirus ITR and the packaging signal followed by the AAV-vector cassette and a duplicate of the adenoviral packaging signal and ITR in reverse orientation (Figure 1, bottom). The hybrid vector is devoid of all viral genes, thus eliminating toxic effects and the elicitation of cellular immune responses. The spontaneous formation of the small hybrid vector genome ΔAd.AAV1 requires the presence of two intact AAV ITRs and does not occur with partly deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette.

Hybrid vectors containing different transgenes:

To construct a hybrid vector with a transgene that can be detected in situ the SEAP/neo expression unit in Ad.AAV1 is replaced by the *E. coli* β-galactosidase gene. This hybrid vector is named ΔAd.AAV1. During generation of the corresponding plasmid constructs the AAV ITR sequences tend to rearrange and abolish their functional properties. This problem can be circumvented by using low copy number plasmids as cloning vectors grown in bacteria strains depleted for all recombination proteins (e.g.JC811). Furthermore, the intactness of both AAV ITRs after each cloning step can be examined for characteristic endonuclease digestion. Recently, another hybrid vector ΔAd.AAV1Nori has been generated which contains the neo gene under the control of both the simian virus 40 (SV40) early promoter and the transposon 5 (Tn5) promoter for expression in human and bacterial cells, as well as the p15A bacterial replication origin with the direction of the leading strand DNA synthesis opposite that of neo gene transcription. Thus, SNori can be used for G418 selection of integrated vector in eukaryotic cells as well as for rescue of vector together with flanking host DNA after integration. The recovered plasmids can be propagated in *E. coli* under selection with kanamycin due to the bacterial origin and the neo gene. SNori containing vectors allow a rapid estimation of total integration events based on the number of G418 resistant colonies. Moreover, vector DNA together with flanking chromosomal DNA can be rescued as plasmids from single G418 resistant clones and can be used for sequencing to determine integration junctions. Both hybrid vectors are produced at a titer of about

3×10^{12} genomes per ml. The ratio of genome titer to transducing particles for ΔAd.AAVBG is ~200:1 based on β-Gal expression.

DISCUSSION

ΔAd.AAV1 could spontaneously form during adenovirus replication. Another possible mechanism of ΔAd.AAV1 formation is based on the unique mechanism by which adenovirus replicates its genome (van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin) (see Figure 1). Ad DNA replication is initiated by the TP/pTP (terminal protein) that binds to specific sites within the ITRs on both ends of the linear genome and catalyzes, in complex with Ad pol, the binding of the 5' CTP, the first nucleotide of the daughter strand. DNA synthesis proceeds in a continuous fashion to the other end of the genome (Figure 1A). Only one of the DNA strands serves as template. One of the replication products is a single-stranded DNA that circularizes through annealing of its self-complementary ITRs. The resulting duplex "panhandle" has the same structure as the termini of the duplex viral genome that allows the binding of pTP and the initiation for synthesis of a complementary strand using the single-stranded "pandhandle" molecule as template (Figure 1C). In the case of Ad.AAV1, the Ad pol synthesizes the single strand of the adenoviral genome starting from the left Ad ITR until it reaches the second AAV ITR. During synthesis of the second AAV ITR a certain percentage of the single-stranded molecules form a loop hybridizing to the complementary region within the first AAV ITR that was replicated earlier, allowing Ad pol to use the same viral DNA strand to read back towards the left ITR (Figure 1B). The resulting "panhandle" structure can be resolved in a similar way as a full-length intermediate shown in Figure 1C, generating a double stranded, linear molecule with the above described structure that can be packaged into Ad virions. The ratio of viral DNA to protein concentration in purified ΔAd.AAV1 particles is comparable to that obtained from Ad.AAV1 particles. This indicates that despite the smaller size, only one ΔAd.AAV1 genome is packaged, resulting in particles with a lighter buoyant density (~1.32 g/cm^3). Electron microscopy demonstrates the icosahedral shape of ΔAd.AAV1 particles (Figure 2). Staining with uranyl acetate causes the central viral cores to appear

electron dense. ΔAd.AAV1 virions have only a spotted luminal dark staining as expected with only one 5.5kb genome being packaged per capsid.

B. *In vitro* ΔAd.AAV1 production:

Characteristics of deleted adeno-AAV vectors (ΔAd.AAV):

A number of experiments to clarify the mechanisms of ΔAd.AAV genome formation are carried out. Specifically, the presence of two intact AAV ITRs flanking a reporter gene cassette is required for the effective formation of ΔAd.AAV genomes. This process does not occur with partially deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette. Furthermore, in vitro transduction studies are performed with different genome titers of ΔAd.AAV1, Ad.AAV1, and Ad.AAV1-Δ2ITRs, (lacking the two AAV ITRs) which determine the number of G418 resistant colonies that formed after 4 weeks of selection (Table I).

ΔAd.AAV1 is routinely produced at a high titer (5 x 10^{12} genomes per ml with $>10^4$ produced genomes per 293 cell) and at a high purity with less than 0.1% contaminating full length Ad.AAV1 genomes by a technique normally used for amplification and purification of recombinant adenovirus.

In vitro transduction studies with hybrid vectors on CD34+ cells and erythroleukemia cells:

In order to test whether the hybrid vectors allow for gene transfer into cell types, that have to be targeted for sickle cell therapy, infection/transduction studies are performed using CD34+ enriched human bone marrow cells, derived from mobilized peripheral blood and the human erythroleukemia cell line K562 which express ε and γ globin genes.

METHODS

Cell Culture:

SKHep1 cells (HTB-52, American Type Culture Collection, Rockville, MD), an endothelial cell line derived from human liver [Heffelfinger, S.C., et al., 1992, *In vitro Cell Dev. Biol.* 28A, 136-4-142], are grown in high-glucose Dulbecco's modified Eagle medium with 10% fetal calf serum. SKHep1 cells are analyzed for integrated AAV provirus by Southern analysis of genomic DNA using the AAV1 wild type genome obtained from pAAV/Ad (Samulski, R. J., et al. 1989. *Journal of Virology* 63:3822-3928) (gift from David Russell, University of Washington) as a probe. No specific bands are detected in undigested genomic SKHep1 DNA or after digestion with HindIII. For viral infection, confluent cells are incubated with different viral doses for 2 hours, followed by intensive washing. For G418 selection, 24h after infection with ΔAd.AAV1, SKHep1 cells are trypsinized and plated at different dilutions under G418 selection (900μg/ml active compound, Boehringer-Mannheim, Germany). G418 containing culture medium is changed every 3 days. The number of colonies with $>10^4$ cells is counted after 4 weeks of selection and divided by the number of initially seeded cells. This ratio is used to express the integration frequency of ΔAd.AAV1. Single colones are obtained by limiting dilutions of infected cells in 96 well plates. Colonies are expanded to $1x10^6$ cells in the presence of G418. Immunofluorescence analysis for adenoviral proteins expressed in SKHep1 cells 3 days post-infection is performed as described earlier [Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791].

RESULTS

293 cells are infected with the first generation vector Ad.AAV1. During replication of Ad.AAV1, the small ΔAd.AAV1 genome forms spontaneously and is packaged into adenovirus capsid. At 36 hours after infection cells are harvested and virus is released by several cycles of freeze/thawing. The mixture of Ad.AAV1 and ΔAd.AAV1 particles in the cell lysate is then separated by ultracentrifugation in a CsCl step gradient. Due to its lighter buoyant density, the band containing the ΔAd.AAV1 particles is clearly separated (0.8cm distance) from the band containing full-length virus (Lieber, A., et al. 1999. *J*

Virol 73:9314-24). ΔAd.AAV1 is purified further by an additional CsCl equilibrium gradient and is stored in 10mM Tris pH7.5, 10% glycerol, 1mM $MgCl_2$ in 80°C. In total, the production of $2x10^{13}$ (genome) particles of ΔAd.AAV1 requires less than 3 hours of work. All functions for ΔAd.AAV1 replication and particle formation are provided from Ad.AAV1 genomes amplified in the same cell. The efficiency of vector production measured on a genome-per-cell-basis is comparable or higher than labor-intensive, newer techniques for rAAV production, which have not yet been proven to be reliable. The estimated ratio of transducing/genome titer for ΔAd.AAV1 is 1:200 (based on SEAP expression at day 3 post-infection), whereas for the average rAAV preparation, it is in the range of $1:10^3$ to $1:10^4$. $1x10^5$ confluent SKHep1 cells are infected with different MOIs of rAAV1 (stock: $1x10^{10}$ genomes per ml), ΔAd.AAV1 (stock: $5x10^{12}$ genomes per ml), Ad.ADAV1 (stock: $1x10^{13}$ genomes per ml), and AdAAV1.2ITR (stock: $9x10^{12}$ genomes per ml), in a volume of 100ml 24 hours after infection, cells are washed, trypsinized, and plated at different dilutions. G418 is added 24 hours after plating and selection is performed for 4 weeks. G418 resistant colonies contain on average $>5x10^4$ cells (at least 16 cell divisions). A significant number of small colonies visible at 2 weeks post-infection do not survive continued selection, probably due to episomal vector expression. Cells infected with first-generation adenoviruses with MOIs greater than $1x10^4$ develop CPE during the first week of selection. The rAAV titer is not high enough to perform infection studies with MOIs greater than 10^4. The colony formation is expressed as percentage of the number of colonies after selection to the number of cells initially seeded for selection (Table I).

TABLE I

Formation of G418 resistant colonies after infection with hybrid viruses in comparison with rAAV.

MOI (genomes per cell)	Formation of G418 resistant colonies in % (SEM) (after 4 weeks of selection)

	rAAV1	Ad.AAV1	Ad.AAV1	Ad.AAV1 2ITRs
10^1	0	0	0	0
10^2	0	0	0	0
10^3	2.7 (1.6)	1.3 (1)	5.4 (3.0)	0
10^4	90.8 (7.0)	48.0 (8.9)	12.9 (7.2)	0
10^5	N/A	93.1 (5.4)	3.8 (2.1)	0
10^6	N/A	100	0	0
10^7	N/A	100	0	0

N = 3 (SEM is indicated in parentheses.)

K562 cells are infected with different MOIs of ΔAd.AAVBG (1-10^8 genomes per cell):

Three days after infection, the total number of viable cells (based on Trypan blue staining) and the percentage of infected cells (based on X-Gal staining) are determined for all MOIs. The results are presented in Figure 4A.

Initial integration studies:

K562 cells are incubated with ΔAd.AAVSNori at an MOI of $2x10^5$ genomes per cell and the colonies that formed after 4 weeks of G418 selection are counted in 96 well plates. G418 resistant colonies contain on average $>5x10^4$ cells which means that the original cell underwent at least 16 cell divisions.

Infection studies with Ad.AAVBG (1-10^8 genomes per cell) on CD34+cells:

Cell infection on CD34+are as described for K562 cells. CD34+ cells are cultured in IMDM supplemented with 20% FCS, kit ligand (stem cell factor-SCF) (100ng/ml), and IL-3 (100ng/ml). Since a number of reports suggest that specific cytokines like GM-CSF or M-CSF which induce stem cell differentiation can stimulate integrin expression and may therefore affect internalization of Ad5 vectors, infection rates are compared with

Ad5 based hybrid vectors on CD34+ cells cultured with and without pre-stimulation with GM-CSF (50ng/ml) or M-CSF (50U/ml). The number of infected cells is counted based on X-Gal staining at day 3 after infection. To test for dose-dependent toxicity, viable cells are counted (based on trypan blue exclusion) at day 3 post-infection. Furthermore, whether high viral doses affect the ability of CD34+ cells to differentiate in methyl cellulose colony assays in presence of IL-3 and SCF is analyzed. The results are expressed as viable cells/X-Gal positive cells vs MOI (see Figure 4B).

DISCUSSION

The above data demonstrate that ΔAd.AAV transduces stably an immortalized human cell line with a low frequency comparable to rAAV, however, transduction rates could be scaled up to 100% by using greater MOIs of ΔAd.AAV1, which is produced at higher titers than rAAV1. In contrast to infection with the first-generation vector, Ad.AAV1, infection with ΔAd.AAV1 is not associated with dose-dependent cytotoxicity because no viral proteins are expressed from these vectors in transduced cells. Furthermore, viral proteins present in the incoming ΔAd.AAV1 particles are not problematic in the dose range use. The comparison of transduction rates of ΔAd.AAV/Ad.AAV1 with the vector lacking AAV ITRs, Ad.AAV-Δ2ITRs, supports the hypothesis that the presence of two intact AAV ITRs is crucial for hybrid vector integration.

The data demonstrate that the leukemia cell line can be infected at ~90% efficiency with Ad5 based hybrid vectors at MOIs $2x10^5$ genomes per cell without significant toxic side effects. However, this dose is still ~100 times greater than the dose necessary to infect 100% of HeLa cell, hepastoma cells, primary hepatocytes and other cell lines generally considered as permissive for Ad5 vector infection.

Since viral DNA in cells infected with $2x10^4$ genomes (or 100 transducing particles per cell) should be lost after 7 cell divisions, the presence of G418 resistant cells in the observed colonies suggests that ΔAd.AAVSNori genomes are integrated into or stably associated with the host genome. Based on the number of G418 resistant colonies one

out of 25,000 ΔAd.AAVSNori genomes integrates stably into K562 cells. This is in agreement with the results obtained earlier with ΔAd.AAV1 in SK Hep1 cells.

The maximal dose used for infection of CD34+ cells ($1x10^8$) results in X-Gal staining of only ~10% of cells independently of GM-CSF/M-CSF. This demonstrates the obvious inability of Ad5 to infect CD34+ cells and is probably caused by the absence of specific receptors and/or integrins on the cell surface. CD34+ cells tolerate a large range of viral doses ($1-10^7$) without obvious effects on cell viability and total cell number. This is not surprising because in order to develop toxic side effects adenovirus has to enter the cell and express viral genes. Hybrid vectors can be produced at titers of $5x10^{12}$ genomes per ml. Thus, the maximal MOI that can be used for infection (of 10^4 cells) is ~$5x10^8$ (in 100μl storage buffer). Based on the infection studies with ΔAd.AAVBG this dose may not be sufficient to efficiently transduce CD34+ cells and to obtain an appreciable number of G418 resistant colonies.

C. *In Vivo* Properties Of ΔAd.AAV1:

Viral DNA is labeled with BrdU during virus amplification to investigate cellular/nuclear vector uptake in situ. For transduction studies, confluent SKHep1 cells (a human endothelial cell line) are infected with 2000 genomes ΔAd.AAV1 or Ad.AAV1 per cell. BrdU tagged viral DNA is detected in 100% of nuclei at 3 hours post-infection for both viruses indicating efficient cellular and nuclear uptake of hybrid virus DNA.

RESULTS

The ΔAd.AAV1 vector transduces a cell in vitro forming G418 resistant colonies with an efficiency of 17 or 58%, after infection with an MOI of $1x10^3$ or $1x10^4$ genomes per cell, respectively. Approximately $2x10^4$ ΔAd.AAV1 genomes are required to yield one stable transfectant. Since all stable colonies contain integrated ΔAd.AAV1 vector DNA, this number reflects the minimal integration frequency of ΔAd.AAV1 in SKHep1 cells which is comparable with that from rAAV (Rutledge, E. A. et al., 1997, *Journal of Virology,*

71:8429-36). The number of G418 resistant colonies does not necessarily represent the total frequency of integration events because not all integrated copies express neomycin phosphotransferase, due to chromosomal position effects or incomplete integration.

The absence of adenoviral gene products in ΔAd.AAV1 transduced cells at day 3 post-infection is demonstrated by immunofluorescence with antibodies to the major late proteins (hexon, fiber) and early proteins (DBP.E4-orf6). Expressed adenoviral proteins are detected only in cells infected with Ad.AAV1. The fact that cells infected with ΔAd.AAV1 do not express potentially cytotoxic adenoviral proteins is important.

While an MOI of 1×10^4 genomes per cell of the first generation vector Ad.AAV1 induce cytopathic effects in SKHep1 cells at day 3 p.i., no toxic side effects are observed when SKHep1 cells are infected with ΔAd.AAV1 at a dose of up to 1×10^8 genomes per cell. Since the transduction efficiency is clearly dose dependent, ΔAd.AAV1 (which can be produced at titers of $>5 \times 10^{12}$ genomes/ml) is able to stably transduce Ad5 permissive cell lines or tissues with a 100% efficiency without associated toxicity.

Southern analysis indicates that ΔAd.AAV1 integrates randomly as head-to-tail tandem repeats into the host cell genome via the right AAV ITR, whereas the other junction with the chromosomal DNA is variable and occurs somewhere within the transgene cassette. In order to confirm the integrated status of ΔAd.AAV1 DNA, high-molecular-weight chromosomal DNA is separated by pulse field gel electrophoresis (PFGE), followed by Southern analysis with a SEAP specific probe (Figure 3). Undigested DNA from control SKHep1 cells give an endogenous SEAP signal that co-migrates with chromosomal DNA just below the well (lanes 1 and 5). No high-molecular weight episomal forms of ΔAd.AAV1 DNA are detected, whereas a distinct 35 kb band is visible in DNA from SKHep1 cells isolated 3 days after infection with first generation adenovirus, Ad.AAV1 (lanes 4 and 13). Digestion with EcoRI reveals the 4.4kb fragment, which is specific for integrated tandem copies of the AAV cassette (lanes 8 and 12). To eliminate the possibility that chromosomal DNA is trapped in the well, DNA samples are digested with intron-encoded endonucleases PI-SceI or I-CeuI (Gibco-BRL, Grand Island, NY) with a

sequence specificity or more than 11bp or 9bp respectively. Digestion with PI-SceI yields a >2mb endogenous SEAP signal in SKHep1 cells (lane 2) and an additional signal in the range of ~1mb in G418 resistant colonies transduced with ΔAd.AAV1 (lane 7). I-CeuI digestion results in a smear between 250-1000 kb in ΔAd.AAV1 transduced SKHep1-cells (lanes 10, 11) indicating random integration, whereas a high-molecular weight band specific for the endogenous SEAP gene is observed in control SKHep1 cells (lane 9).

One day after intraportal infusion of $1x10^{12}$ ΔAd.AAV1 genomes in C57Bl/6 mice, BrdU labeled vector genomes can be detected in 85% hepatocytes (Lieber, A., et al. 1999. *J Virol* 73:9314-24). Hepatocellular DNA analysis performed at 2 months post-infusion reveals ΔAd.AAV1 DNA integrated with an average of 0.5 copies per cell into the mouse genome (Lieber, A., et al. 1999. *J Virol* 73:9314-24). To assess potential side effects of intraportal ΔAd.AAV1 infusion, serum glutamic pyruvic transaminase (SGPT), a sensitive marker for hepatocellular injury, is measured for 7 consecutive days post-infusion in combination with histological analysis of liver sections. No significant elevation in SGPT levels, or histological abnormalities are detected after intraportal infusion of $1x10^{12}$ or $1x10^{13}$ ΔAd.AAV1 genomes, whereas infusion of the same dose of full-length Ad.AAV1 vector is associated with severe hepatoxicity or fatal outcome. This suggests that the dose of ΔAd.AAV1 administered to mice can be increased to obtain higher transduction efficiencies in vivo without adverse side effects, which is not possible for first generation adenoviruses. Importantly, ΔAd.AAV1 transduced quiescent hepatocytes in vivo, which suggests that integration of hybrid vector DNA may not require cell proliferation. Recently, more detailed in vivo transduction studies with Ad.AAV1 and ΔAd.AAV1 have been performed in Balb/c mice to study whether the absence of adenoviral gene expression in cells infected with ΔAd.AAV1 can avoid an anti-viral immune response and can prolong vector persistence. In this mouse strain, vector DNA is cleared from the liver at 4-6 weeks after infusion with first generation adenoviruses, mostly due to a CTL response against viral proteins produced in transduced cells. Vector DNA is analyzed by genomic Southern Blot of hepatic DNA at 12 weeks

after infusion of $1x10^{12}$ genomes Ad.AAV1 or ΔAd.AAV1. At this time point, no vector specific signal is detectable in hepatic DNA from mice infused with the first generation vector Ad.AAV1, while ~0.3 copies of ΔAd.AAV1 genomes per cell are present in livers of mice that received the hybrid vector, again indicating the superior in vivo properties of the hybrid vector.

D. Effects of Rep Coexpression on ΔAd.AAV Integration

Rep expression after plasmid transfection:

In order to test whether Rep expression enhances site-specific integration of ΔAd.AAV1 in human cells, a series of Rep expression plasmids are constructed.

METHODS

The Rep ORF 68/78 (nt 285-2313) including the internal p19 and p40 promoters is obtained from pAAV/Ad (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia) by digestion with BsaI/BsrI. This fragment deleted for the AAV p5 promoter is cloned via adapter linkers under RSV or PGK promoter in front of the bovine growth hormone polyadenylation signal (bPA) into pAd.RSV or pAd.PGK (Lieber, A., and Kay, M.A., 1996, *J. of Virology*, 70, 3153-3158; Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) correspondingly.

RESULTS

The resulting plasmids (pRSVrep, pPGKrep) are transfected into 293 cells or SKHep1 cells, most of the Rep proteins expressed from the heterologous promoters (RSV or PGK) are Rep 68 and Rep 78, while transfection of the rep gene under aP5 promoter (pAAV/Ad) results in predominant Rep 52/40 expression. Thus, transfection of pRSVrep and pPGKrep is more pronounced suggesting a strong transactivation of AAV promoters

by Ela which is produced in 293 cells. This result indicates that minimum expression of rep proteins is necessary to avoid interference with adenovirus replication.

Rep-mediated site-specific integration of ΔAd.AAV1.

The potential for site-specific integration is an important characteristic of the novel Ad.AAV vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

A preliminary test can be performed to confirm the functional activity of Rep 68/78 expressed from pRSVrep to mediate site-specific integration of ΔAd.AAV1 (Figure 5 and 6). Human SKHep1 cells are transfected with pRSVrep or control plasmid (pRSVbGal (Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) (transfection efficiency was ~20%), followed by infection with ΔAd.AAV (2000 genomes per cell). Three days after infection, cells are trypsinized, embedded in agarose, lysed in situ, digested with I-CeuI (an intron-encoded endonuclease with a recognition sequence of more than 10nt), subjected to pulse file gel electrophoresis in 1% agarose gel, and analyzed by Southern Blot. Hybridization with a probe covering the AAVS1 integration site (1.7kb EcoRI/BamHI fragment from the chromosome 19 locus (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia)) reveals an AAVS1-specific band (~240kb) in I-CeuI digested DNA from cells after control plasmid transfection (pCo) + ΔAd.AAV1 infection. An additional signal in the range of 280kb appears in rep expressing cells infected with ΔAd.AAV1

(pRSVrep + VAd.AAV1) indicating a site-specific insertion into the AAVS1 site in a certain percentage of cells. The presence of vector DNA in this 280kb band is confirmed by rehybridization of the same filter with a transgene (SEAP) specific probe. Randomly integrated ΔAd.AAV1 vector appears as a diffuse SEAP signal in the range 280-680kb (pCo+ΔAd.AAV1, pRSVrep+ΔAd.AAV1). The specific ~1.9 mb band on blots hybridized with the SEAP probe represents an I-CeuI fragment containing the endogenous human SEAP gene.

Incorporation Rep 68/78 function into hybrid vectors to stimulate site-specific integration

Rep overexpression inhibits adenovirus DNA replication, prohibiting the generation of rep expressing Ad vectors using conventional strategies. To solve this problem, significant Rep 68/78 expression from the hybrid vector in virus producer (293) cells must be prevented while maintaining transient Rep expression in target cells (HSC) to mediate site-specific integration. Our hypothesis is that the specific structure of the ΔAd.AAV hybrid virus can be used to bring the rep gene 68/78 into a transcriptionally active position under control of a HSC specific promoter only at late stages of virus replication in 293 cells. This will allow amplification of the hybrid vector in 293 cells, generating high titer virus which activates the incorporated Rep 68/78 functions only in HSC. The general outline of our strategy to produce Rep expressing hybrid vectors is illustrated in Figure 7. The rep/transgene cassette is assembled based on the left-hand shuttle plasmid used for recombinant adenovirus production. The gene encoding Rep 68/78 is cloned in 3'□5' orientation in front of a transgene expression cassette flanked by AAV ITRs. Between the transgene cassette and the right AAV ITR an HSC-specific promoter is inserted with direction towards the adenoviral E2, E3, and E4 genes. The recombinant genome is produced by recombination in E.coli and transfection into 293 cells generates virus (Ad.AAV-rep). The specific structure of ΔAd.AAV with duplicated sequences flanking the AV ITRs is used to bring the rep gene into a transcriptionally active position under control of a HSC specific promoter only during late stages of viral DNA replication in 293 cells. During amplification of Ad.AAV-rep, the smaller genome ΔAd.AAV-rep is formed and packaged into particles, which can be separated by ultracentrifugation in CsCl gradients. The specific structure of ΔAd.AAV-rep brings the

rep gene into 5'↔3' orientation in relation to the HSC specific promoter, allowing rep transcription in target cells. After transduction of HSC with purified ΔAd.AAV-rep particles, rep expression is activated and mediates rescue of the AAV-ITR/transgene cassette from the adenoviral vector backbone and site-specific integration. The hypothesis is that Rep-mediated integration into AAVS1 occurs via the right or both AAV/ITRs causing the rep gene to become separated from the hepatocyte-specific promoter once the vector is integrated (Figure 7). Therefore, rep expression should be only transient without critical cytotoxic side effects on the host cell.

Promoters that can regulate rep expression:

Potential candidate promoters to drive rep expression with high specificity for HSC and minimal activity in 293 cells are the 454nt CD34 promoter (Krause, D.S., et al., 1997, *Experimental Hemotology*, 25, 1051-1061; Yamaguchia, Y. et al., 1997, *Biochimica et Biophysica Acta.*, 1350:141-6), the 300nt HS 40 enhancer (Chen, H.L., et al., 1997, *Nucleic Acids Res.* 25, 2917-2922) or a 3kb CD34 enhancer (May, G. et al., 1995, *EMBO J.*, 14:564-74) in combination with an initiator, or the HIV LTR. An optimal promoter is selected based on studies of transient reporter gene expression after plasmid transfection in 293 cells and hepatocytes. All promoters to be tested are cloned in front of the human α_1-antitrypsin (hAAT)- bovine growth hormone polyadenylation signal (bPA) into the adenoviral shuttle plasmid pCD2 (pAd.-hAAT). Promoter activity can be tested in transient plasmid transfection assays in CD34+ and 293 cells. The promoter with the highest hAAT levels in CD34+ or K562 cells and the lowest hAAT expression in 293 cells is selected for further studies. If high background expression in 293 cells from these promoters is seen, insulators to shield HSC-specific promoters from the Ela enhancer which is still present in Ad shuttle plasmids can be utilized.

Rep genes:

The large Rep 68/78 proteins are sufficient to mediate rescue and site-specific integration. Unregulated Rep 52 and Rep 40 expression from the AAV p19 promoter

located within the ORF of Rep 68 and 78 must be prevented because production of these smaller Rep proteins in 293 cells will affect cell viability and adenoviral DNA synthesis. To do this, constructs obtained from Surosky et al., containing a mutated Rep 52/40 start codon to express Rep 68 and 78 individually under CMV promoter can be used. The 293 cells transiently expressing Rep68 or Rep 78 from these constructs can be coinfected with ΔAd.AAV1 (infection 24 hours after pCMVRep transfection, MOI $2x10^5$ genomes/cell). Three days after ΔAd.AAV1 infection, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE as described earlier. Efficient Rep mediated excision of the AAV cassette and site-specific integration without flanking adenoviral sequences are expected and the plasmids pCMVRep68 or pCMVRep78 can be used as a source for the corresponding rep genes and clone them into hybrid vectors.

Vectors:

The rep/transgene cassette can be assembled based on pXCJL (Microbix, Toronto). A set of control hybrid vectors can be generated with the AAV-ITR-transgene cassette only without the rep gene. The recombinant Ad.AAV-rep genome can be generated by recombination of the left hand shuttle plasmids with pCD1, a pBHG10 (Microbix, Toronto) derivative, which contains the Ad5 genome deleted for the E1/E3 regions in $recA^+$ E. coli (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Compared to the standard technique based on plasmid recombination in 293 cells, this approach has the advantage that plaques with recombinant virus appear 3 times faster and the production of illegitimate recombinants is minimized. This allows efficient viral DNA amplification and packaging to occur before Rep expression reaches levels that are potentially inhibitory for adenoviral replication. The critical variables in maximizing the output of the vector deleted for all adenoviral viral genes are the initial multiplicity of infection and the time of harvesting. These parameters can be optimized for production of ΔAd.AAV-rep hybrid vectors. A number of ΔAd.AAV vectors can be constructed incorporating rep gene. Cryptic promoter and enhancer elements present in the 5'-342nt of the adenoviral genome can interfere with transgene expression from the heterologous promoters. This is crucial for the strategy to avoid rep expression from ΔAd.AAV-rep genomes in 293 cells.

To ensure efficient transgene expression, insulator fragments such as the chicken beta-globin insulator can be used with a selected promoter, constitutive or inducible.

Rep protein co-packaging:

As an alternative to producing hybrid vectors containing the rep 68/78 gene, studies are designed to see whether Rep protein can be co-packaged into ΔAd.AAV capsids and whether these co-packaged Rep molecules are sufficient to mediate rescue and site-specific integration of the AAV-ITR-transgene cassette. Our hypothesis is that the Rep 68/78 binds to the Rep binding site (RBS) present in double-stranded ΔAd.AAV genome and that this complex is co-packaged into adenoviral capsids which are spacious enough to accommodate extra proteins. Based on protein/DNA ratio analysis performed previously in purified particles that only one 5.5kb ΔAd.AAV1 genome is packaged per capsid. This is confirmed by electron-microscopy of ΔAd.AAV1 particles, which reveals only spotted electron-dense staining associated with viral cores and extended free luminal space (see Figure 2).

293 cells are transfected with plasmids expressing Rep 68/78 under the CMV promoter and the kinetics of rep expression is determined by Western Blot with cell lysates collected at different time points after transfection. Next, these 293 cells are infected with Ad.AAV (MOI 1, 10, 100 pfu/cell) at specific time points after transfection of Rep plasmids depending on the Rep expression kinetics (e.g. 3, 6, 12, 24 . . . hours after transfection). It is important to time Ad.AAV infection exactly because viral DNA replication must be taking place or finished before Rep production reaches peak levels. In general, adenovirus DNA replication in 293 cells (infected with MOI 10) is maximal at 18 hours post-infection, followed by production of structural proteins, packaging of viral genomes, and breakdown of cellular membrane structures (which is concluded ~36-48h p.i.) (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia; van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin). Viruses are collected 48h after infection and banded by CsCl ultracentrifugation. Viral material from purified bands corresponding to

ΔAd.AAV is lysed, DNAse-treated (to liberate DNA associate Rep) and subjected to immunoprecipitation-Western Blot with Rep specific antibodies to detect co-packaged Rep. Based on theoretical calculations assuming that two Rep molecules bind per Ad genome, ~1-10ng Rep proteins is expected from Lysates of 10^{10} particles, which is within the range of detectability by Western Blot. Alternatively, co-packaged Rep may be detected based on its functional activity to mediate rescue and site-specific integration of the AAVITR transgene cassette. To test whether functional Rep protein is co-packaged into hybrid vector particles, CsCl purified ΔAd.AAV1 particles generated in 293 cells co-expressing Rep after Ad/AAV1 infection (ΔAd.AAV1+Rep) can be used for transduction studies. Three days after ΔAd.AAV1+Rep infection of the human cell line K562, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE. If efficient Rep-mediated site-specific integration of excised AAV cassettes is successful, then other ΔAd.AAV+Rep hybrid vectors with β-Gal and SNori as transgenes can be produced.

Integration studies with Rep vectors in erythroid cells:

The hypotheses behind the rational of a rep-expressing hybrid vector (ΔAd.AAV-rep) are: (1) transient Rep co-expression from ΔAd.AAV-rep vectors can enhance site-specific vector integration in human cells and (2) integration occurs via the AAV ITR(s) without the rep gene, which is placed outside the AAV cassette, thus eliminating rep expression upon vector integration. To test hypothesis 1, transduction frequencies of ΔAd.AAV/rep versus ΔAd.AAV vectors can be compared based on the formation of G418 resistant colonies and quantify site-specific integration events at different time points after infection of human and mouse cells by PFGE and PCR. To test hypothesis 2, the structure of integrated vector in transduced cell populations and single clones can be delineated by Southern analysis and by sequencing of vector/chromosomal DNA junctions. These studies can be performed with ΔAd.AAV-rep,ΔAd.AAV, and ΔAd.AAV+Rep (copackaged protein) in human K562 or HEL (for AAVS1 integration) and mouse MEL cell lines.

Cells infected with ΔAd.AAV-SNori, ΔAd.AAV-SNori+Rep or ΔAd.AAV-Snori-rep can be subjected to G418 selection. The number of G418 resistant colonies determined after 4 weeks of selection in relation to the number of initially infected cells. The selection process for colonies that did not survive continued selection due to potential rep-mediated cytotoxcicity or episomal vector expression can be monitored. If rep expression from ΔAd.AAV-SNori rep does not affect cell viability and proliferation, then more G418 resistant colonies should appear in ΔAd.AAV-SNori-rep and ΔAd.AAV-Snori+Rep. The structure of integrated vector can be determined by Southern Blot and sequencing of integration junctions.

To uncover a potential selection bias against rep producing cells after transduction with ΔAd.AAV/rep. site-specific and random vector integration events can be quantitated in cellular DNA isolated from cell populations at different time points after infection (e.g. 0.5, 1, 3, 7. 14 days). To do this, the techniques based on PFGE-Southern can be utilized. It is expected that the signal(s) for AAVS1-specific integration in ΔAd.AAV/rep infected human cells increases during the first days after infection and then remains constant over time.

In a separate study, the integration status of vector DNA (analyzed by PFGE or PCR) and the number of integrated copies (analyzed by Southern Blot) with the expression level of β-galactosidase in single clones transduced with β-Gal hybrid vectors (ΔAd.AAV-BG, ΔAd.AAV-BG+Rep, or ΔAd.AAV-BG-rep) can be correlated. Together with data obtained in the studies described in the Specification, this allows assessment of whether transcriptional silencing is associated with site-specific vector integration into the AAVS1 site.

It is not clear a priori whether the specific Rep function for vector rescue, concatemerization, and integration can efficiently occur in non-S-phase or non-dividing cells. To test whether ΔAd.AAV^{fx} , ΔAd.AAV or ΔAd.AAV-rep/+Rep vectors can integrate into non-dividing cells, transduction studies in cell cycle arrested cell cultures can be performed as described earlier.

DISCUSSION

The establishment of stable cell lines expressing Rep 68/78 at detectable levels is not possible, which is probably due to rep mediated cytotoxicity. Therefore, it is not possible to perform long-term transduction studies (e.g. G418 selection or studies in single clones) in combination with ectopic rep expression. Moreover, due to the inhibitory effect of rep on adenovirus replication, it is currently not possible to generate adenoviral vectors expressing rep under the RSV or PGK promoter.

Taken together, this indicates that co-expressed Rep may stimulate site-specific transgene integration.

E. A Detailed Study Of Transduction/Integration Of Hybrid Vectors In Erythroid Cell Lines:

In order to improve transduction and integration frequencies of the hybrid vectors into erythroid cell lines, a detailed study comparing various hybrid vectors have to be carried out as described below. The transduction studies are performed in K562 cells which is considered to be an adequate model to study gene transfer vehicles into erythroid cells (Floch, V., et al., 1997, *Blood Cells, Mol. and Diseases.*23, 69-87). The optimal vectors should be able to integrate into the cellular genome with a high frequency, determined by Pulse field gel electrophoresis (PFGE) and Southern blot as described in Example 4. In addition, the results from the following studies will serve to evaluate whether a given hybrid vector needs to be modified for site-specific integration in the host genome.

Sequencing of integration junctions:

The ultimate proof for vector integration is the sequencing of junctions between SNori vector DNA and chromosomal DNA. Furthermore, this clarifies the question whether the AAV ITRs represent the substrate for integration. Specifically, DNA from clones with

known ΔAd.AAVSNori integration structure (analyzed by Southern Blot) digested with EcoRI, which does not cut within the SNori cassette. The resulting fragments are circularized and transformed into a specific E. coli strain (according to the protocol described by Rutledge and Russell (Rutledge, E. A. et al., 1997, *Journal of Virology*, 71:8429-36)). Kanamycin resistant bacterial clones should contain the integrated SNori cassette. Flanking chromosomal DNA in rescued plasmids can be sequenced with primers specific to the transgene.

To confirm vector integration in a small number of transduced cells, genomic DNA is extracted and digested with EcoRI. EcoRI fragments are ligated to linkers containing a specific primer binding site and are then digested with NotI, religated and propagated in *E. coli*. Plasmid DNA from a representative number of bacterial clones is sequenced to determine the vector/chromosomal DNA junctions.

Dose dependent toxicity:

In order to test that the transduction frequence is dose-dependent and ΔAd.AAV vectors, which are devoid of all adenoviral genes, could be used to infect cells at higher doses with less cytotoxicity than first generation adenovirus, K562 cells are infected with different MOIs ($1\text{-}10^8$) of ΔAd.AAVBG and the first generation vector Ad.AAVBG (which contains the same β-Gal expression cassette). At day 4 post-infection, the total number of cells, the percentage of viable cells (based on trypan blue exclusion) and the percentage of X-Gal positive cells are counted. A fraction of infected cells are quantified for β-Gal expression using the Galacto-Light kit. The level of transgene expression is expected to be comparable between the two vectors. K562 cells are predicted to tolerate higher doses of ΔAd.AAVBG better than Ad.AAVBG which express viral genes.

Integration frequency with and without G418 selection:

In order to investigate the integration frequency of the different vectors and to confirm that AAV ITRs present in double-stranded adenoviral DNA genomes can mediate vector

integration with a frequency comparable to rAAV vectors. integration studies are performed based on the formation of G418 resistant colonies with ΔAd.AAVSNori, AdSNori, Ad.SNoriITR, and rAAVSNori after infection with $2x10^5$ and $2x10^6$ genomes per cell (Fig. 8). After infection, cells are plated in 96 well plates under limiting dilution and selected with G418 to estimate the frequency of formation of G418 resistant colonies. Another set of cells is plated without G418. A representative number of clones (w/ and w/o G418 selection) are expanded to $>10^6$ cells (after 3-4 weeks of culture) and analyzed for the presence of viral DNA by Southern Blot as well as PFGE analysis to discriminate between episomal vector DNA and vector genomes stably associated with chromosomal DNA. This allows us to estimate the integration frequency of the different vectors, to assess the effect of G418 selection on integration, and to consider position effects on neo expression in calculating the total integration frequency. Integrated vector copies with a frequency of at least $1x10^{-4}$ is predicted only for ΔAd.AAVSNori and rAAVSNori. The total number of colonies may be lower in both the first generation vectors, Ad.AAVSNoriITR and Ad.SNori, due to the toxic effects of expressed adenoviral proteins; however, a higher integration frequency is predicted for the vector containing the AAV ITRs (Ad.AAVSNoriITR).

Kinetics of integration:

Compared to rAAV, the double-stranded nature of entering ΔAd.AAV genomes provides more protection against degradation. Furthermore, the synthesis of transcriptionally active double-stranded intermediates from single-stranded genomes, which is considered a limiting step in rAAV transduction, is not required in ΔAd.AAV transduction. Thus, the lag phase between infection and expression seen with rAAV vectors, which is causally linked to double-strand synthesis/integration may be shorter or absent in infections with ΔAd.AAV vectors. Furthermore, it was demonstrated earlier that a 9kb mini-adenoviral genome packaged into adenoviral particles is only short lived and completely degraded by day 3 post-infusion. In contrast, transduction with the 5.5kb ΔAd.AAV1 (Figure 1) genome allows for long-term expression, suggesting that either

AAV ITRs can stabilize the viral genome as an episome until it is integrated or integration occurs shortly after infection.

The status of vector DNA can be examined in K562 cells at different time points after infection with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, or rAAVSNori (MOI $2x10^5$). Infected cells are harvested at 1 hour, 5 hours, 1 day, 3, 7, and 14 days after infection and chromosomal DNA is analyzed by PFGE followed by hybridization with a transgene specific probe. This technique allows us to distinguish between episomal vector DNA, which appears as a distinct 5.0kb band and integrated DNA. Furthermore, extra chromosomal high-molecular weight vector concatemers can be detected. In the case of random integration, after digestion of chromosomal DNA with I-CeuI or PI-SceI, vector-specific signals in the range of 1-2mb should be seen. The intensity of episomal and integrated vector signal is quantified for each time point using phosphoimager analysis. This gives information about the kinetics of hybrid vector integration in a population of infected K562 cells and the intracellular stability of hybrid vector genomes.

Structure of integrated vector DNA and integration junctions with chromosomal DNA:

ΔAd.AAV1 integrates as concatemer/s randomly into host DNA as shown previously. How many vector copies are present in one concatemer and whether the extent and the kinetics of tandem-formation are dose dependent still remain unclear. Another unanswered question is how ΔAd.AAV integrates: whether one or both ITRs are involved, whether the integrated ITRs are still intact, and whether adenoviral sequences integrate as well. These issues are important for the strategy to include rep genes into the hybrid vector genome. Moreover, if intact AAV ITRs are present within integrated vector copies, helper virus (adenovirus or HSV) infection in vivomay mobilize the integrated AAV-ITR vector cassette and affect stability of transgene expression.

To answer these questions, K562 cells can be infected with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, and rAAVSNori at MOIs $2x10^5$, $2x10^6$, or $2x10^7$ genomes per cell. Infected cells are plated in 96 well plates in the presence or the absence of G418. The

latter is included because G418 may cause amplification of integrated vector DNA (Rutledge, E. A. et al., 1997, *Journal of Virology,* 71:8429-36). Genomic DNA from isolated clones can be analyzed by regular Southern Blot as described in the Examples Section to confirm the presence of vector concatemers and calculate the number of integrated vector copies. More informative is the sequencing of integrated vector copies and their junctions with chromosomal DNA. The structure of integration junctions can be delineated using the role of AAV ITRs in vector integration and the extent of insertional mutagenesis after transduction. This data provides information about the potential risks of hybrid vector used in clinical trials.

Transduction of cell cycle arrested cells:

The ultimate target for the hybrid vectors described in the Specification are quiescent hematopoietic stem cells. We hypothesize that the double-stranded nature of ΔAd.AAV genomes and specific nuclear import mechanisms may allow for the transduction of non-dividing cells. This is in part supported by the transduction studies with ΔAd.AAV1 in quiescent hepatocytes in vivo. To confirm this data, primary fibroblasts can be forced to enter the G_0 phase by serum/growth factor starvation before infection with the hybrid vectors according to a protocol described by Russell (Russell, D. et al., 1995, *PNAS,* 92:5719-23). Cells are maintained for three days after infection under serum/growth factor deprivation. At this time point, genomic DNA is isolated and analyzed for integration events by PFGE in comparison with growing cells. Another series of integration studies can be carried out on K562 cells arrested in the G_1/S phase of the cell cycle with aphidicolin (added 1 day before and maintained several days after infection with hybrid vectors - depending on the integration kinetics studies described earlier). To investigate whether DNA damaging agents increase the transduction frequency of hybrid vectors, cell-cycle-arrested K562 cells or primary fibroblasts can be treated with cisplatinum or ^{3}H-thymidine prior to virus infection according to a protocol described by Alexander and Russell (Alexander, I. E. et al., 1994, *J. Virol.,* 68:8282-87; Russell, D. et al., 1995, *PNAS,* 92:5719-23). Furthermore, the effect of chromosomal DNA decondensation on the transduction efficiency of hybrid vectors can be studied in arrested

cells after treatment with puromycin, staurosporin, Hoechst 3328, distramycin, or vandate.

F. Improvements in ΔAd.AAV Production and Purification

To inhibit packaging of full-length genomes a modified form of I-Sce I, a yeast mitochondrial intron-endonuclease with a non-palindromic 18-bp recognition sequence is expressed in 293 cells. Constitutive expression of this enzyme in mammalian cells is not toxic, possibly due to either the lack of I-SceI sites in the genome or sufficient repair of them (Rouet P. et al, 1994, *PNAS*. 91:6064-8). The yeast I-Sce I is modified with an SV40 T-antigen nuclear localization signal and an optimal Kozak sequence to enhance its functionality in mammalian cells (Rouet P. et al, 1994, *PNAS*, 91:6064-8). For another yeast endonuclease it was shown that a recognition site within an transduced Ad genome was efficiently (30% of all transduced genomes) when expressed in human A549 cells. Importantly, the expression of E4 ORF6 and ORF3 expressed from the transduced Ad genome inhibited double-strand break repair mediated by the endonuclease (Nicolas, A. L. et al, 2000, *Virology*, 266:211-24). This is consistent with the observations by others where these E4 proteins prevent concatemerization of the viral genome (Boyer, J. et al, 1999, *Virology*, 263:307-12). Based on this, packaging of full-length virus containing a I-SceI recognition site is reduced in 293 cells constitutively expressing I-Sce I. The 18mer I-Sce site is inserted into the E3 region of the Ad.IR vectors. These vectors are generated and amplified in 293 cells followed by a large-scale infection of 293 cells expressing I-SceI. Alternatively, an expression cassette for the endonuclease XhoI is inserted into the E3 region of Ad.IR or Ad.AAV vectors. The XhoI gene will be modified for optimal function in mammalian cells. Vectors expressing XhoI are generated and amplified in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Nelson, J. E. et al, 1997, *J. Virol.*, 71:8902-7), which mediates the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG. This protects the viral and cellular genome from XhoI cleavage. Methylated Ad vectors are produced at high titers. ΔAd.AAV vectors are then obtained by large-scale infection of 293 cells with the Ad.AAV-XhoI vectors. At this stage the viral

genome is not methylated and is digested at the XhoI sites. XhoI sites present within the transgene cassette are deleted by site-directed mutagenesis without altering the amino acids sequence. (XhoI is accumulated only at late stages in virus replication and should act only upon a large part of Ad DNA when replication is completed. In addition, ultracentrifugation optimizes the separation between ΔAd.IR and ΔAd.IR particles (Blague, C. et al., <u>2000</u>, *Blood*, 95:820-8).

EXAMPLE II

MODIFIED FIBER PROTEIN

A. Test the Infectivity of Different Human or Animal Serotype on Human Bone Marrow Cells.

Since the amino acid sequence of the fiber knob region varies considerably among the ~50 known serotypes, it is thought that different adenovirus serotypes bind to different cellular receptor proteins or use different entry mechanisms (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14; Defer, M., et al., 1993, *J. of Virology*, 64, 3661-3673). Although most adenoviruses contain RGD motifs in the penton base proteins, there are a number of serotypes (e.g. Ad 40, 41) without this conserved sequence. These types may use integrin □v-independent pathways for virus internalization (Davison, A.J., et al., 1993, *J. Mol. Biol.* , 234, 1308-1316; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14). To test whether other Ad serotypes can infect stem cell subpopulation present in human bone marrow, studies with a series of different human Ad serotypes and animal viruses can be performed (see Table II). As a means to verify efficient transduction with Ad serotypes, viral DNA is tagged before infection and the presence of viral genomes in the nuclei of transduced cells is investigated. Furthermore, whether viral DNA is replicated in transduced cells can be analyzed as indirect proof for early viral gene expression. A direct detection of expressed viral proteins is impossible due to the unavailability of antibodies against all the serotypes included in this study. Simultaneously with the infection assay, transduced

human bone marrow cells can be analyzed for morphological and immunohistochemical features characteristic of HSC or progenitor subpopulations. For retargeting, serotypes which are able to infect CD34+ subsets of bone marrow cells at the lowest MOI are selected. As the next step, the fiber gene is PCR-cloned from serotypes with potential HSC/CD34+ tropism and inserted into standard shuttle plasmids för Ad5 vector generation replacing the Ad5 fiber gene using an E. coli recombination system (Figure 9).

METHODS

<u>*Cells and viruses:*</u>

HeLa (human cervix carcinoma, ATCC CCL-2.2), CHO (chinese hamster ovary, ATCC CCL-61), K562 (human hematopoietic, ATCC 45506), HEp-2 (human larynx carcinoma, ATCC CCL-23), 293 (human embryonic kidney, Microbix, Toronto Canada) cells were maintained in DMEM, 10% FCS, 2 mM glutamine, and Pen/Strep. Culture media for CHO cells was supplemented with 200μM asparagine and 200μM proline. Human CD34+-enriched bone marrow cells were purified from peripheral blood after mobilization using MiniMACS VS^+ separation columns (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. Aliquots were stored in liquid nitrogen. Sixteen hours before the experiment, cells were recovered from the frozen stock and incubated overnight in IMDM media, supplemented with 20% FCS, 10^{-4} M β-mercaptoethanol, 100 μg/ml DNaseI, 2 mM glutamine, 10 U/ml IL-3, and 50 ng/ml stem cell factor (SCF) or 2 ng/ml thrombopoietin (Tpo). The purity of CD34+ preparations was verified by flow cytometry and was consistently greater than 90%.

<u>*Flow cytometry:*</u>

Adherent cells (CHO, HeLa) grown in non-tissue culture treated 10 cm dishes (Falcon, Franklin Lakes, NJ) were detached by treatment with 1mM EDTA and washed three times with wash buffer (WB), consisting of PBS supplemented with 1% FCS. Cells

grown in suspension (K562, CD34+) were washed three times with WB. After washing, cells were resuspended in WB at 2 x 10^6 cells/ml. 2 x 10^5 cells were incubated in WB for 1 h at 37°C with monoclonal antibodies specific for α_v-integrins [L230, ATCC: HB-8448, (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795) (1/30 final dilution), CAR [RmcB (Bergelson, J. M., et al. 1997. *Science*. 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology*. 62:1647-1652) (1/400 final dilution)], or BrdU [(Amersham, Arlington Heights, IL) (1/100 final dilution)]. Subsequently, cells were washed with WB, and incubated with fluorescein isothiocyanate (FITC)-labeled horse anti-mouse IgG antibodies [(Vector Labs., Burlingame, CA) (1/100 final dilution)] or phycoerythrin (PE)-labeled goat anti-mouse IgG antibodies [(Calbiochem, La Jolla, CA) 1:100 dilution] for 30 min at 4°C. After incubation with secondary antibodies, cells were washed two times with WB and 10^4 cells per sample were analyzed in duplicate by flow cytometry.

For the analysis of CD34 and c-kit expression on transduced CD34+-cells and for fluorescent activated cell sorting (FACS), purified human CD34+ cells were incubated with phycoerythrin(PE)-conjugated anti-CD34 monoclonal antibodies (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA) or with PE-labeled anti-CD117 (c-kit) monoclonal antibodies (MAb 95C3, Immunotech, Beckman Coulter, Marseille, France) according to the manufacturer's protocol followed by flow cytometry analysis. All analyses and sortings were performed on a FACStar Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with 488nm argon and 633 nm HeNe lasers. For analysis of c-kit expression and FACS purification of CD34+/c-kit+ cells, SCF was not added to the media during culturing of CD34+ cells.

RESULTS

CAR/α_v-integrin expression on test cells:

It is generally accepted that CD34+ cells possess bone marrow repopulating activity. Therefore, we used human CD34+ cells as the target for our studies towards identifying Ad serotypes with HSC tropism and constructing new viral vectors. Studies were

performed on mobilized, CD34-positive, peripheral blood cells from one donor under conditions which are known to retain CD34+ cells in a quiescent stage (Leitner, A., et al. 1996. *Br.J.Haematol.* 92:255-262; Roberts, A. W., Metcalf, D. 1995. *Blood.* 86:1600-1605). More than 90% of purified cells were CD34 positive by flow cytometry. Furthermore, we included into our Ad tropism studies the cell line K562, which is considered to be an adequate model for studying gene transfer into human hematopoietic cells (McGuckin, et al. 1996. *British Journal of Haematology*. 95:457-460). HeLa cells, which are readily infectible by Ad5, and CHO cells, which are refractory to Ad5 infection (Antoniou, M. et al., 1998, *Nucleic Acid Res.*, 26:721-9), were used as positive and negative control cell lines, respectively.

For Ad5, both, binding to the primary receptor and to $\alpha_3\beta_5$ and $\alpha_\varpi\beta_5$ integrins are important for high efficiency infection of target cells. The expression of CAR and α_v integrins on test cells was analyzed by flow cytometry using monoclonal antibodies against CAR (RmcB (Bergelson, J. M., et al. 1997. *Science*. 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology*. 62:1647-1652)) and α_v integrins (L230 (Roelvink, P. W., et al. 1996. *J. Virology*. 70:7614-7621)) (Figure 10). As expected, nearly all HeLa cells expressed high levels of CAR and α_v-integrins, whereas CHO cells lacked significant CAR and α_v-integrin expression. Fifteen and 77% of K562 cells expressed CAR and α_v-integrins, respectively. Only ~6% of the CD34+ cells used in our studies expressed CAR and 17% were positive for α_v-integrins. Notably, the preparation of CD34+ cells represents a mixture of different cell types. The absent or low expression of primary and secondary Ad5 receptors on non-cycling human CD34+ cells is in agreement with previous reports (Huang, S., et al. 1996. *J. Virology*. 70:4502-4508; Neering, S. J., et al. 1996.. *Blood.* 88:1147-1155; Tomko, R. P., et al.. 1997. *Proc. Natl. Acad. Sci. USA.* 94:3352-3356).

Infection assay using wild-type Ad5 and K562 cells:

The presence of viral DNA in the nucleus of infected cells is an indirect means to demonstrate efficient virus binding, internalization, and nuclear import. Nuclear

localization of the viral genome is a prerequisite for transgene transcription and integration. Two techniques are utilized to tag viral DNA for in situ analysis. To optimize the infection assay, wild-type Ad5 virus and K562 cells which are permissive for Ad5 infection can be used. The first protocol (Challberg, S.S. and Ketner, S. 1981, *Virology* 114, 196-209), is based on ^{32}P-labeling of viral DNA. During amplification of wild-type Ad5 and A549 cells, ^{32}P-phosphate (40μCi/ml) is added to phosphate-free medium. After development of CPE, ^{32}P-tagged virus is harvested, banded in CsCl gradients, and titered on HeLa cells according to standard protocols. To simulate the conditions for infection of human bone marrow cells, K562 cells are incubated in suspension with a MOI of 1, 10, or 100 of ^{32}P-Ad5 for 2, 4, 6, or 8 hours under agitation at 37°C. This covers the time period necessary for adsorption, internalization, and nuclear import. After washing, cells are fixed either transferred to microscopy slides using cytospin or embedded in paraffin and sectioned (according to protocols from VECTOR labs, Burlingham, CA). The latter has the potential advantage that multiple consecutive sections (5μm) of the same cell can be analyzed by different methods (e.g. for ^{32}P tagged viral DNA, for specific histological staining, for immunofluorescence), which allows for correlating infection with a particular cell type present in the bone marrow. Cells are incubated in a Kodak NTB-2 photo emulsion for autoradiography. The exposure time can be optimized to minimize background or non-nuclear localized signals. A dose and time dependent appearance of nuclear silver grains is expected under the optimized conditions. Since ^{32}P-phosphate can label viral proteins as well, a cytoplasmic background signal might appear. To facilitate detection, immunofluorescence with HSC specific antibodies on sections can be performed. As an alternative method, a BrdU-labeling technique for viral DNA can be used (Lieber, A., et al. 1999. *J Virol* 73:9314-24; Lieber, A. et al., 1996, *Journal of Virology*, 70:8944-60). In this case, different amounts of BrdU are added to the A549 culture medium during wtAd5 virus propagation. BrdU labeled viral DNA can be detected with monoclonal antibodies specific to BrdU. The signal can be enhanced using layers of species-specific polyclonal antibodies in combination with biotin/avidin and a fluorescent marker. BrdU tagged viral DNA can be detected on cytospins of bone marrow cells together with cell surface markers by double or triple immunoflourescence.

DISCUSSION

The interaction of selected Ad serotypes with CD34+ cells was tested. As a result of this screening we constructed a first-generation, Ad5-based vector whose fiber was substituted with the fiber derived from Ad35. We demonstrated that this capsid modification allowed for efficient viral transduction of potential HSCs by the corresponding chimeric Ad vectors.

All tropism and transduction studies were performed with non-cycling CD34+ cells, which are thought to include HSCs. The quiescent stage of CD34+ cells purified from mobilized blood is important because induction of cell proliferation is associated with a loss of the ability to reconstitute hematopoiesis and with changes in the spectrum of cellular receptors (Becker, P. S., et al. 1999. *Exp. Hematol.* 27:533-541). It is known that treatment of hematopoietic cells with cytokines or growth factors changes the expression of specific integrins including α_v-integrins, which would ultimately alter the susceptibility of cells to Ad infection or may effect viability of infected cells (Gonzalez, R., et al. 1999. *Gene Therapy.* 6:314-320; Huang, S., et al. 1995. *J. Virology.* 69:2257-2263). Another fact that complicates the interpretation of transduction studies is the extraordinary heterogeneity of CD34+ cells in regards to morphology and function.

B. Screening different adenoviruses to establish tropism to HSC.

The ATCC provides more than 70 different human or animal adenoviruses (see Appendix I). A collection of 15 human serotypes and 6 animal adenoviruses (see Table II) are selected based on the following criteria: (i) availability of the complete genome sequence or fiber sequence from the NIH gene bank (ii) CAR receptor usage absent or unknown, (iii) different subgroups, and (iv) moderate or low tumorigenicity (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia). However, any serotype shown in the Appendix hereto can be used for the invention described. Animal viruses are included in the infectivity assay because this may provide a

means to circumvent the pre-existing humoral immunity against human Ad5 fiber, which represents a critical obstacle for clinical trials with Ad vectors.

METHODS

Viruses:

The following human adenovirus serotypes were purchased from the ATCC: 3 (VR-3), 4 (VR1081), 5 (VR-5), 9 (VR1086), 35 (VR-716) and 41(VR-930). Adenovirus No. VR-716 was purchased from ATCC labeled as serotype 34, however it was found to be serotype 35 upon sequencing of the fiber region. For amplification, the corresponding Ads were infected onto HeLa, 293, or HEp-2 cells under conditions that prevented cross-contamination. Virus was banded in CsCl gradients, dialyzed and stored in aliquots as described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaque titering was performed as follows: Confluent 293 cells plated in 6-well plates were incubated for 24 hours with virus in a total volume of 1ml. Two weeks after infection, plaques were counted on cultures overlayed with 1% agarose/MEM/10% FCS.0

EM studies:

CsCl-banded Ad stocks were thawed and diluted with 0.5% glutaraldehyde. Grids were prepared as described earlier (Mittereder, N., et al. 1996. *J. Virology*. 70:7498-7509). After staining with 2% methylamine tungstate (Nanoprobes, Stony Brook, NY), the carbon-coated grids were evaluated and photomicrographed with a Phillips 410 electron microscope, operated at 80 kV (final magnification 85,000x). For each particular Ad serotype, the number of morphologically deficient viral particles per 100 was counted in five random fields.

RESULTS

Electron microscopy:

Little is known about the stability of particles from serotypes other than Ad5. Since the intactness of viral particles was crucial for comparative interaction studies, virions from the serotypes specified above were analyzed by electron microscopy (EM). EM studies of negative contrast stained Ad suspensions demonstrated that the percentage of defective particles (loss of icosahedral shape or luminal staining) did not exceed 5% indicating that serotype preparations had comparable qualities. Representative EM photographs are shown for Ads 5, 9, and 35 (Figure 11).

Serotype screening:

It is thought that different Ad serotypes bind to different cellular receptor proteins and use different entry mechanisms (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Mathias, P., et al. 1994. *Journal of Virology*. 68:6811-6814). A set of human adenoviruses was obtained from the ATCC to be tested for tropism to CD34+ cells. These included serotypes 3, 4, 5, 9, 35, and 41 representing different subtypes (Table 1). We believed that these serotypes would use different cellular attachment and internalization strategies due to differing lengths of fiber shafts (Chroboczek, J., et al. 1995. *Adenovirus fiber*, p. 163-200. *In* a. P. B. W. Doerfler (ed.), *The molecular repertoire of adenoviruses*, vol. 1. Springer Verlag, Berlin; Roelvink, P. W., et al. 1998. *J. Virology*. 72:7909-7915), the presence or absence of RGD motifs within the penton base, and differing tissue tropism. The relatively little characterized Ad35 was selected because it was found in immunocompromised hosts, particularly in bone marrow recipients (Flomenberg, P., et al. 1994. *Journal of Infectious Diseases*. 169:775-781; Flomenberg, P. R., et al. 1987. *Journal of Infectious Diseases*. 155:1127-1134; Shields, A. F., et al. 1985 New England *Journal of Medicine*. 312:529-533). The latter observations prompted us to believe that bone marrow cells are among the natural reservoirs for Ad35.

TABLE II

Human and animal adenoviruses with potential interest for the invention

Adenovirus	Human/ Group B	Human/ Group D	Human/ Group F	Avian	Bovine	Canine	Ovine	Swine	Mouse
Serotype	<u>3</u>,<u>7</u>,11,16, 21,34,35	8,15,17, 19,28,37	<u>40</u>, <u>41</u>	CELO, EDS	3	1,2	5	4	1

The underlined serotypes use CAR independent pathways for cell entry.

For amplification, the corresponding adenovirus stocks can be infected onto HeLa or A549 cells such that at a given time only one virus type is handled in a separate laminar flow hood and cultured in Hepa-filtered bottles, preferentially in separate CO_2 incubators to avoid cross-contamination. During propagation, viral DNA is tagged using one of the techniques described earlier. Viral DNA can be isolated from purified particles. The XhoI restriction pattern is analyzed for methylated and unmethylated viral DNA by Southern blot using the full genome of the corresponding virus type as a radioactive probe.

DISCUSSION

Although it was reported earlier by slot-blot assay that fiber knobs derived from 2, 9, 4, and 41L can bind to CAR (Roelvink, P. W., et al.. 1998. *J. Virology*. 72:7909-7915), it is not clear whether this binding occurs with an affinity that is physiologically relevant and whether this would confer cell entry. Furthermore, as shown for the Ad5 interaction between the penton and intergrins, a secondary receptor is required to induce virus internalization. We demonstrated that different serotypes interacted differently with the K562 or CD34+ target cells. Ad5, Ad4, and Ad41 were not able to efficiently attach to and be internalized by K562 and CD34+ cells. Although Ad4 belongs to a separate subgroup (E), it is thought that Ad4 represents a natural hybrid between subgroup B and C viruses with a fiber related to Ad5 (Gruber, W. C., et al. 1993. *Virology*. 196:603-611).

Therefore, it was not surprising that Ad4 has binding properties similar to Ad5. The subgroup F serotype Ad41 has been shown to contain distinct fibers, a long shafted and a short-shafted fiber allowing for different cell entry pathways (Tiemessen, C. T., Kidd, A.H. 1995. *J. Gen. Virol.* 76:481-497). The Ad41 penton base does not contain RGD motifs suggesting that this virus may use α_v-intregrin independent pathways for cell entry. However, these features did not improve interaction with CD34+ cells. Ad9, Ad3, and Ad35 did interact with CD34+ cells more efficiently than Ad5. Out of all the serotypes tested, Ad35 demonstrated the most efficient attachment and internalization with K562 and CD34+ cells. Although the short-shafted Ad9 can bind to CAR, it preferentially uses α_v-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Therefore, the low level of α_v-integrin expression on certain subset/s of CD34+ cells may account for the observed susceptibility to Ad9.

C. Attachment and Internalization of the Ad serotypes to K562 and CD34+ cells.

METHODS

Labeling of Ads with [^{3}H]-methyl thymidine:

Serotypes were labeled with [^{3}H]-methyl thymidine as described in detail elsewhere (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Briefly, 5×10^7 HeLa or 293 cells were grown in 175 sq. cm flasks with 15 ml DMEM/10% FCS and infected with wild type adenovirus at a MOI of 50 or higher. Twelve hours post-infection, 1 mCi of [^{3}H]-methyl thymidine (Amersham, Arlington Heights, IL) was added to the media and cells were further incubated at 37°C until complete CPE was observed. Then, cells were harvested, pelleted, washed once with cold PBS, and resuspended in 5 ml PBS. Virus was released from the cells by four freeze-thaw cycles. Cell debris was removed by centrifugation and viral material was subjected to ultracentrifugation in CsCl gradients and subsequent dialysis as previously described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology.* 70:8944-8960). Virus purification and dialysis removed unincorporated radioactivity. Wild type Ad particle concentrations were determined

spectrophotometrically by measuring the OD_{260}, utilizing the extinction coefficient for wild-type Ad5 $\varepsilon_{260} = 9.09 \times 10^{-13}$ OD ml cm virion^{-1} (Maizel, J. V., et al. 1968. *Virology*. 36:115-125). The virion specific radioactivity was measured by a liquid scintillation counter and was always in the range of 1×10^{-5} to 1×10^{-4} cpm per virion. For selected variants, the fiber gene was PCR amplified and sequenced to ensure identity and the absence of cross-contamination.

Viral DNA tagged with methylase and test for replication by genomic Southern blots:

To ultimately confirm transduction, a protocol to detect adenoviral replication in infected cells can be established. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. A site-specific methylation strategy is utilized to monitor viral DNA replication within infected cells (Nelson, J. et al., 1997, *Journal of Virology*, 71:8902-07). Methylation marked adenovirus can be produced by the addition of a methyl group onto the N6 position of the adenine base of XhoI sites, CTCGAG, by propagation of the virus in HeLa or A549 cells expressing the XhoI isoschizomer PaeR7 methyltransferase (PMT) (Kwoh, T.J., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 7713-7717). It is known that methylation does not affect vector production but does prevent cleavage by XhoI. Loss of methylation through viral replication restores XhoI cleavage and can be detected by Southern blots of genomic DNA from infected cells in comparison to native, non-methylated, viral genomes.

Attachment and internalization assays:

These studies were performed based on a protocol published elsewhere (Wickham, T. J., et al. 1993. *Cell*. 73:309-319). In preliminary experiments, we found that labeled Ad5 virions reached equilibrium in attachment to HeLa cells after 45 min at 4°C with an MOI of 400 pfu per cell. For attachment studies, 3.5×10^5 cells were incubated for one hour on ice with equal amounts of [^{3}H]-labeled adenovirus OD particles equivalent to an MOI of 400 pfu/cell for Ad5 in 100 µl of ice-cold adhesion buffer (Dulbeco's modified Eagle's medium supplemented with 2 mM $MgCl_2$, 1% BSA, and 20 mM HEPES). Next, the cells

were pelleted by centrifugation for 4 min at 1000 x g and washed two times with 0.5 ml ice-cold PBS. After the last wash, the cells were pelleted at 1500 x g, the supernatant was removed, and the cell-associated radioactivity was determined by a scintillation counter. The number of viral particles bound per cell was calculated using the virion specific radioactivity and the number of cells. To determine the fraction of internalized [^{3}H]-labeled adenoviral particles, cells were incubated on ice for one hour with the corresponding virus, washed with PBS as described above, resuspended in 100 μl adhesion buffer, and then incubated at 37°C for 30 min. Following this incubation, cells were diluted 3-fold with cold 0.05% trypsin-0.5mM EDTA solution and incubated at 37°C for an additional 5-10 min. This treatment removed 99% of attached radioactivity. Finally, the cells were pelleted at 1500 x g for 5 min, the supernatant was removed, and the protease-resistant counts per minute were measured. This protocol minimizes the possibility that the internalization data were affected by receptor recycling (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795). Nonspecific binding of Ad particles to cells on ice was determined in the presence of 100-fold excess of unlabeled virus. This value routinely represented less than 0.1 % of viral load.

RESULTS

Attachment of Ad particles to target cells and internalization:

The selected serotypes were metabolically labeled with [^{3}H]-thymidine, which is incorporated into viral DNA during replication. Adsorption and internalization can be experimentally dissociated by taking advantage of the observation that at low temperature (0-4°C) only virus cell attachment occurs, whereas internalization requires incubation at higher temperatures. The number of particles adsorbed or internalized per cell was calculated using the virion-specific radioactivity and used to quantify interaction of Ads 3, 4, 5, 9, 35, and 41 with CD34+, K562, HeLa and CHO cells (Figure 12). The serotypes varied significantly in their ability to attach to and to be internalized by the different cell lines. For Ad5, the degree of attachment to the cell lines tested correlated with the level of CAR expression. In CHO cells, which were previously shown to be refractory to Ad5

infection, the level of attachment and internalization was about 50-70 viral particles per cell. This number was hereafter assumed negative in terms of susceptibility of a given cell type for Ad5. Interaction of the other serotypes with CHO cells was not significantly higher indicating that corresponding receptor/s were absent on CHO cells. All serotypes tested interacted with HeLa cells; with Ad3 and Ad35 being the most efficient variants. The presence of distinct Ad3 and Ad5 receptors on HeLa cells was demonstrated previously (Stevenson, S. C., et al. 1995. *J. Virology*. 69:2850-2857). Ads 4, 5, and 41 did not bind to K562 cells. In contrast, Ad9 as well as the members of subgroup B, Ad3 and Ad35, efficiently interacted with K562 cells with Ad35 having the highest number of adsorbed and internalized particles. Compared to Ad5, about 25 times more Ad35 particles were attached and three-forth of these were internalized by K562 cells. Viral interactions with CD34+ cells were generally weaker. Among the serotypes tested, only Ad9 and Ad35 were significantly internalized by non-cycling CD34+ cells. Internalization of Ad9 and Ad35 was, respectively, four and eight times more efficient than for Ad5 particles. The number of Ad35 virions internalized by CD34+ cells was almost half of that seen for Ad5 in HeLa cells, which can be readily infected with Ad5 based vectors.

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into HeLa, 293, and CHO cells:

Hela and 293 cells expressing high level of primary and secondary receptors for human adenoviruses are used as a positive control for virus attachment and internalization. As a negative control CHO cells are used. CHO cells do not express the primary adenoviral receptor at a detectable level, and are therefore refractory for adenoviral infection. For attachment studies, these adherent cell lines are detached from 10 cm dishes with PBS-EDTA solution (without Ca2+ and Mg2+), washed three times with ice-cold PBS, resuspended in adhesion buffer, and incubated with viruses as described above in the Examples section. As expected, all adenoviral serotypes tested are efficiently attached to and internalized into Hela cells (Table III) (Figure 13). Adenoviruses serotypes 3, 5, 35, 41, but not 9, are efficiently attached to and internalized by 293 cells. In contrast, poor

attachment and internalization of most adenovirus serotypes are observed with CHO cells. The level of attachment on CHO is about 50-70 virus particles per cell for adenoviruses serotypes 5 and 41, 115 virus particles per cell for adenovirus type 3 and about 180 particles per cell for adenovirus serotypes 9 and 35. For further analysis, numbers >300 viral particles per cell are assumed as positive and <70 viral particles per cell as negative in terms of susceptibility of a particular cell line for efficient adenoviral transduction.

TABLE III

Comparative analysis of attachment and internalization of Ad5 and Ad9 to cell lines, expressing different amounts of CAR and αυβintegrins.

Cell line	CAR expression	αυβ-integrin expression	Ad9 (attached/ internalized)	Ad5 (attached/ internalized)
HeLa	++	++	426/370	550/500
CHO	-	++	300/300	70/50
293	++	++	20/20	1950/1750
Y79	+++	-	190/140	1200/1100
K562	-	+	320/230	60/50
Erythrocytes	?	?	420/-	68/-

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into human CD34+ bone marrow cells and K562 erythroleukemia cell line:

Previous studies showed that the human erythroleukemia cell line K562 can be transduced with Ad5-based adenoviral vectors at very high MOIs. As shown in Figure 14, only about 60 viral particles per cell of adenovirus serotype 5 are attached to and even fewer particles are internalized into these cells at a MOI of 400. In contrast to Ad5, about 320 viral particles per cell of Ad9 and about 1500 viral particles per cell of Ad35 are

attached to and about two-thirds of them are internalized into K562 cells (Figure 14B). Human unstimulated CD34+-enriched bone marrow cells obtained from frozen stocks are incubated overnight in growth medium without cytokine stimulation. The next day, the number of viable cells is calculated. For attachment studies, cells are washed three times with ice-cold PBS, resuspended in adhesion buffer and incubated with adenoviruses. Among the adenoviral serotypes tested, only adenovirus particle of Ad9 (about 150 viral particles per cell) and Ad35 (about 320 viral particles per cell) are able to attach to unstimulated CD34+ cells on the level, compared to Ad 5 (only 60 viral particles per cell). Four-fifths of these virus particles are able to be internalized by the cells. Interestingly, upon stimulation of CD34+ cells with GM-CSF and EPO/TPO for two weeks, attachment and internalization of Ad9 viral particles are significantly increased (up to 300 particles per cell). At the same time, the transient stimulation of cells with GM-CSF for two days could not increase the level of viral attachment to the cells.

Based on the above finding that Ad35 serotype is able to attach and internalize into CD34+ cells most efficiently among several serotypes tested, serotype Ad35 was selected for further studies. As described in Appendix II, a chimeric vector (Ad5 GFP/F35) containing the short-shafted Ad35 fiber sequence in an Ad5 capsid was able to target a broad spectrum of CD34+ cells in a CAR/integrin independent manner.

DISCUSSION

In summary, from all the serotypes tested, Ad9, Ad3, and Ad35 demonstrated the most efficient attachment to and internalization with K562 and CD34+ cells. Based on adsorption/internalization data, Ad9 and Ad35 as representatives for subgroups D and B were selected for further tropism studies.

D. Characterization of Ad vector replication in K562 and CD34+ cells.

Comparative analysis of Ad5, and Ad9 and Ad34 to infect and to replicate in 293, K562 and CD34+ cells. The ability of the Ad9 fiber knob domain to recognize the same

primary receptor on the cell surface as Ad5 with comparable affinity was described earlier. Thus, the finding that Ad9 viral particles can only poorly attach to 293 cells is rather unexpected. In order to find out how the attachment and internalization data reflect the biological activity of adenoviruses of different serotypes, the stocks of Ad5, Ad9 and Ad35 are characterized in more detail by electron microscopy, plaque assay on 293 cells, and quantitative replication assay in K562 and CD34+ cells.

METHODS

Quantitative replication assay:

1×10^5 CD34+ or K562 cells were infected in 100μl of growth media with different MOIs of Ad5, 9, or 35 which had been amplified in 293 cells, expressing the XhoI DNA methyltransferase isoshizomer PaeR7 (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). After 2 hours of incubation at 37°C, the cells were centrifuged at 1000 x g for 5 min, the virus-containing medium was removed, the cells were resuspended in 100μl of fresh media, and then they were incubated at 37°C until harvesting. At 16 hours post-infection for K562 cells, or 36 h post-infection for CD34+ cells, 5 μg of pBS (Stratagene, La Jolla, CA) plasmid DNA was added as a carrier which could also be used as a loading control. Genomic DNA was extracted as described previously (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). One-fourth of purified cellular DNA (equivalent to 2.5×10^4 cells) was digested with HindIII, XhoI, or with HindIII and XhoI together at 37°C overnight and subsequently separated in a 1% agarose gel followed by Southern blot with chimeric Ad5/9 or Ad5/35 DNA probes. The chimeric probes, containing sequences of Ad5 and Ad9 (Ad 5/9) or Ad5 and Ad35 (Ad 5/35), were generated by a two-step PCR amplification using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) and viral DNA from purified particles as a template. The following primers were used for PCR (Ad5 sequences and nucleotide numbers are underlined): Ad5F1 – <u>(nt: 32775-32805)</u> 5'-<u>GCC CAA GAA TAA AGA ATC GTT TGT GTT ATG</u>-3'; Ad5R1 – <u>(nt: 33651-33621)</u> 5'-<u>AGC TGG TCT AGA ATG GTG GTG GAT GGC GCC A</u>-3'; chimeric Ad5/9F – (nt: <u>31150-31177</u>, nt: 181-208) 5'-<u>AAT</u>

GGG TTT CAA GAG AGT CCC CCT GGA GTC CTG TCA CTC AAA CTA GCT GAC CCA -3'; chimeric Ad5/9R – (nt: 32805-32775, nt:1149-1113) 5'- CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC TTC ATT CTT GGG CGA TAT AGG AAA AGG-3; chimeric Ad5/35F – (nt: 31150-31177, nt: 132-159) 5'-AAT GGG TTT CAA GAG AGT CCC CCT GGA GTT CTT ACT TTA AAA TGT TTA ACC CCA-3', chimeric Ad5/35R (nt: 32805-32775, nt: 991-958) 5'-CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC ATT TTA GTT GTC GTC TTC TGT AAT GTA AG-3'. Nucleotide numbers are given according to the sequences obtained from the NCBI GenBank (accession No. M73260 / M29978 for Ad5, X74659 for Ad9, and U10272 for Ad35). After the first amplification, the 968 bp-long Ad9, a 859 bp-long Ad35 DNA fragments corresponding to the fiber genes, and a 876 bp-long Ad5 fragment corresponding to the Ad5 E4 region (located immediately downstream of Ad5 fiber gene) were purified by agarose gel electrophoresis. To generate chimeric DNA probes, amplified Ad5 DNA was mixed with Ad9 or Ad35 fragments obtained during the first step of PCR, and subjected to a second PCR amplification using Ad5/9F or Ad5/35F primers and the Ad5R1 primer. The resulting Ad5/9 or Ad5/35 chimeric DNA fragments (see Fig. 15) were purified and their concentrations were measured spectrophotometrically. Corresponding chimeric DNA fragments were loaded as concentration standards on agarose gels or labeled with [^{32}P]-dCTP and used as probes for Southern analysis. The number of viral genomes per DNA sample was calculated after quantitative Phospho-imager analysis. In preliminary experiments, no preferential hybridization of chimeric DNA probes to DNA of any particular viral serotype was detected.

RESULTS

Replication of selected serotypes in K562 and CD34+ cells:

Adsorption/internalization studies do not ultimately prove viral transduction, a process often defined as gene transfer that allows for viral or heterologous gene expression in host cells. Intracellular trafficking, including endosomal lysis, transport to the nucleus,

and nuclear import of the viral genome, depends on structural capsid proteins and thus, varies between different serotypes (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Miyazawa, et al. 1999. *J. Virology*. 73:6056-6065). We believed that analysis of viral gene expression would be a means to verify successful nuclear import of viral genomes and that this would be a good criterion for selection of serotype/s able to efficiently infect our target cells. To do this, we used a protocol, which allows for the detection of Ad replication in infected cells. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. We utilized a site-specific methylation strategy to monitor viral DNA replication within infected cells (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). Methylated Ad serotypes were produced by the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG, during propagation of the viruses in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Kwoh, T. J., et al. 1986. *Proc.Natl.Acad.Sci. USA*. 83:7713-7717) (293 PMTcells). Loss of methylation through viral replication restores Xho I cleavage and can be detected by Southern blots of Xho I-digested genomic DNA from infected cells.

Ad replication studies were performed in K562 and CD34+ cells with Ad9 and Ad35, in comparison to Ad5. For replication studies, the infectious titer (in pfu/ml) and genome titer (in genomes per ml) were determined (by plaque assay on 293 cells or by quantitative Southern blot, respectively) for methylated and unmethylated Ad5, Ad9, and Ad35 (Table 2). The ratio of pfu to genome titer was comparable for methylated and unmethylated virus demonstrating that DNA methylation had not altered transduction properties. About 85% of (Ad5, 9, and 35) virus used for infection was methylated as calculated based on the intensity of fragments specific for methylated and non-methylated viral DNA present in the viral load (Fig. 15). The numbers of genomes detected after adsorption (1 hour, 0°C) or internalization (2 hours 37°C) correlated well with studies shown in Fig. 12. Ad9 and Ad35 interacted more efficiently than Ad5 with K562 and CD34+ cells. Dose-dependent replication studies in K562 and CD34+ cells were performed with the same genome numbers of Ad5, 9, and 35 (Fig. 15). The replication rate was measured based on the ratio of methylated to demethylated viral

DNA after infection with different MOIs (2100, 420, and 105 genomes per cell). In K562 cells, efficient replication (100% conversion from methylated to unmethylated DNA) was detected for Ad5 at MOI >/= 2100, for Ad9 at MOI >/=420, and for Ad35 at MOI >/=105. This demonstrated that Ad35 transduced K562 cells with the highest efficiency. In CD34+ cells, the replication rate was 100% for Ad5 and 31% for Ad9 after infection with MOI 420. Although methylated Ad35 viral DNA was present in CD34+ cells, viral replication was undetectable for Ad35. In summary, while viral replication studies in K562 cells confirmed data obtained for Ad5, 9, and 35 adsorption and internalization, there was a discrepancy between earlier results and the poor replication of Ad9 and, particularly, Ad35 in CD34+ cells. As outlined later, replication analysis in heterogeneous cell populations, like CD34+ cells, may not allow for definitive conclusions on tropism of a particular serotype.

Taking all the screening data together, Ad9 and Ad35 emerged as the variants with the strongest tropism for K562 and CD34+ cells. It is thought that Ad9 can bind to CAR, however, it preferentially uses α_V-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology*. 70:7614-7621). This entry strategy may be not optimal for efficient infection of CD34+ cells as only less that 17% of them express α_V-integrins (Fig. 10). Therefore, we decided to concentrate on Ad35 as a source for heterologous fiber to be used for construction of a chimeric vector based on an Ad5 backbone.

TABLE IV

Results from the infectivity assay which determines the optical particle-to-PFU (OPU/PFU) ratio using 293 cells

Virus	OPU (A260)	PFU	OPU/PFU ratio
Ad5	1.4×10^{12}	1.06×10^{11}	13
Ad9	4.61×10^{11}	2.6×10^{8}	1773

DISCUSSION

Viral replication studies in K562 cells confirmed the data obtained for Ad5, 9, and 35 adsorption and internalization. However, there was a discrepancy between the interaction data and the replication data in CD34+ cells where Ad9 replicated only poorly and no replication was seen for Ad35. Ad replication is only initiated upon the production of a critical threshold of early viral proteins, which in turn, is directly dependent on the number of viral genomes present in the nuclei of infected cells. Therefore, the outcome of replication studies may be affected by the rate of nuclear import of viral genomes, by the activity of viral promoters, and/or the intracellular stability of viral DNA/RNA. These parameters may vary, on one hand, between different subsets of CD34+, and/or, on the other hand, between different Ad serotypes. In conclusion, the viral replication analyses performed with different Ad serotypes in CD34+ cells may not predict the actual transduction properties of chimeric vectors based on Ad5 backbone. This implies that attempts to produce gene transfer vectors based on Ad genomes other than Ad5 should be exercised with caution.

Recently, an Ad serotype screening strategy was used to identify variants with tropism for primary fetal rat CNS cortex cells or human umbilical vein endothelial cells. The optimal serotype (Ad17) was selected based on immunohistochemistry for hexon production 48 hours after infection (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540). However, this approach is problematic because, at least in our hands, antibodies developed against Ad5 hexon did not cross-react with other serotypes. Also, hexon is expressed only after onset of replication. As outlined above, the kinetics of intracellular trafficking, viral gene expression, and replication significantly vary between serotypes (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Miyazawa, et al. 1999. *J. Virology*. 73:6056-6065).

In addition to being the most efficient serotype in terms of interaction with CD34+ cells, Ad35 is also interesting because it interacts with receptor/s different from the Ad5 and Ad3. Ad35 and Ad5GFP/F35 attachment was not inhibited by Ad5 or anti-CAR antibodies suggesting that Ad35 binding was CAR independent. First, Ad5 did not compete with Ad35 and Ad5GFP/F35 during internalization and infection indicating that

$\alpha_{\square}\beta_{3/5}$ integrins are not involved in viral entry. Second, function-blocking antibodies against α_v-integrins did not compete with Ad35 and Ad5GFP/F35 for internalization into K562 cells, whereas these antibodies did inhibit Ad5 internalization. And third, in contrast to Ad5 based vectors, GFP expression after infection with Ad5GFP/F35 was not restricted to α_v–integrin-expressing CD34+ cells. From these facts, we conclude that infection with Ad35 and the chimeric Ad5GFP/F35 vector does not involve α_v-integrins. In this context, the presence or absence of RGD motifs within Ad35 penton base remains to be determined by sequencing the corresponding genome region. Cross-competition assays demonstrated that Ad35 and Ad5GFP/F35 bind to a receptor that is different from the Ad3 receptor. Although Ad3 and 35 belong to the same subgroup, they have been divided into two DNA homology clusters, B1 and B2; the amino acids composing their fibers are only 60% homologous. Furthermore, the target tissues for both viruses are different; Ad3 can cause acute respiratory infections, whereas Ad35 is associated with kidney infection (Horwitz, M. S. 1996. *Adenoviruses,* p. 2149-2171. *In* B. N. Fields, Knipe, D.M., Howley, P.M. (ed.), *Virology*, vol. 2. Lippincott-Raven Publishers Inc., Philadelphia). Therefore, it was not surprising to see that Ad3 and Ad35 recognize different receptors.

In conclusion, Ad35 and the chimeric vector enter the cells by a CAR- and α_v-integrin independent pathway. We believe that Ad35 and the chimeric vector binds primarily to its fiber receptor and that this interaction is sufficient to trigger internalization. On the other hand, Ad35 internalization may involve cellular proteins other than α_v-integrins. These membrane proteins can overlap with those for Ad3 internalization and represent $\beta 2$ integrins, which protrude more from the cell surface than α_v-integrins (Huang, S., et al. 1996. *J. Virology*. 70:4502-4508).

According to EM studies of negative contrast-stained adenoviral suspensions, the percentage of deficient particles for all adenoviral serotypes tested does not exceed 5%. However, plaque assays reveal that the ability to form plaques in 293 cells is significantly different for tested serotypes. The optical particle-to-PFU (OPU/PFU) ratio obtained is 13 for Ad5, which is in good agreement with the previously estimated ratio for this

adenoviral serotype. Importantly, this ratio is about three times higher for adenovirus serotype 35 and more than 150-fold higher for adenovirus serotype 9. Furthermore, quantitative Southern blot using chimeric Ad5/9 and Ad5/35 DNA probes is used to determine the ratio between the genome and transducing titer. This study confirms the data obtained by plaque assay. Quantitative replication assay of these adenoviruses in K562 and CD34+ cells also confirms the ability of Ad9 and Ad34 to more efficiently attach to these cell types. The replication of viral genomes is observed for Ad9 and Ad34 at lower MOIs of infection, compared to Ad5. In conclusion, the data obtained for different serotypes in attachment and internalization are in good agreement with the infectivity data in target cells.

E. Attachment and internalization of different adenoviral serotypes into primary dendritic cells, JAWSII, MCF-7 and REVC cells.

As a proof of principle, the serotype screening strategy can be employed for other important target cells which are refractory to Ad5 infection.

RESULTS

RECV cells are endothelial cells which have to be targeted for approaches that are aimed to gene therapy of restenosis, atherosclerosis, inflammation etc. MCF-7 cells are breast cancer cells isolated from liver metastases which are important targets for tumor gene therapy. The human adenovirus serotypes 3, 5, 9, 35 and 41 are tested to see whether they can attach to and can be internalized by mouse primary dendritic cells, JAWSII cells, MCF-7-human breast cancer cells and REVC endothelial cells. None of the adenoviral serotype tested can efficiently attach to primary dendritic cells. Adenovirus serotype 3 is able to efficiently attach to REVC endothelial cells (about 400 virus particles per cell are attached and about 300 are internalized). In comparison, only 50 Ad5 particles are able to attach to and even fewer are internalized in these REVC. The human breast cancer cells (MCF-7) are previously shown to be refractory to Ad5

infection at low MOIs. However, Ad3 and more efficiently, Ad35 attach to and internalize into MCF-7 cells.

DISCUSSION

The data presented herein indicate that different human adenovirus serotypes recognize different cellular receptors and can therefore infect cell types that are refractory to Ad5 infection. There are adenoviral serotypes that can more efficiently attach and internalize than Ad5 for human CD34+ cells, REVC, K562 and MCF-7 cells. This finding provides a basis for the construction of chimeric adenoviral vectors which are Ad5 vectors containing receptor ligands derived from other serotypes.

F. Infection studies on primary human bone marrow cells.

Since established erythroleukemic cell lines do not represent an adequate model for the ultimate hematopoietic stem cell that has to be targeted in patients in order to achieve long-term reconstitution with genetically modified cells, normal primary human bone marrow cells are used for the initial infection/retargeting studies.

RESULTS

In a first set of tropism studies with different Ad serotypes, whole bone marrow cell suspensions can be used without preselection. This is advantageous because the tropsim of various adenovirus serotypes or genetically retargeted vectors can be analyzed on a broad spectrum of progenitor subpopulations representing myeloid, erythroid, megakaryocytic, lymphoid, dentritic, and monocytic lineages. For short term (< 5 hours) infection studies, bone marrow suspensions can be cultured in IMDM supplemented with 10% FCS, β-mercaptoethanol, and 10u/ml IL-3 for ensuring cell viability.

Mononucleated cell assays:

Mononucleated bone marrow cells can be incubated with MOI 1, 10, 100, or 1000 pfu/cell of the various adenovirus types for a short time. Paraffin sections or cytospins of infected bone marrow cells can be analyzed for nuclear-localized, labeled viral DNA. BrdU labeling can be visualized by immunoflouresence with anti-BrdU antibodies; ^{32}P-tagged viral DNA can be detected by incubation with photo-emulsion. In addition, the same cell material can be analyzed for morphology after specific histo-staining (e.g. Wright-, Hemo3 staining). If required, commercially available antibodies can be used to specific cell surface markers conjugated directly to different fluorochromes (FITC (green), TRIT., RPE, (red), RPE-Cy5, AMCA (blue)) to completely characterize infected bone marrow subpopulations. Colocalization of BrdU-labeled viral DNA (e.g. as FITC signal) with membrane markers signifying infection of specific cell types can be demonstrated; for example, potential stem cells/early progenitors ($CD34^+$, $CD38^-$), megakaryocytes (CD4la+), eryhthroid cells (glycophorin A+), dentritic cells (CDla+), monocytes (CD14+), or myeloid cells (CD15+), etc. The morphological analysis of infected bone marrow subsets gives a first information whether specific adenovirus serotypes can target primitive cell types.

DISCUSSION

Since the different wild-type adenoviruses do not express a uniform marker gene and do not integrate and since detection of tagged viral DNA cannot be done on live cells, it is not possible, at this point, to characterize infected cells for clonogenic or repopulation capacities. Therefore, adenovirus serotypes for retargeting studies are selected, based on their ability to infect in vitro purified CD34+ cells at low MOIs. This subset of bone marrow cells is known to contain long-term reconstituting cells. Infection studies with different adenovirus serotypes can be repeated on purified CD34+ cells (cultured in IMDM +10% FCS, β-mercaptoethanol, and 10 units/ml IL-3) as described above. Purification of CD34+ cells can be performed by direct immunoadherence on anti-CD34 monoclonal antibody-coated plates or on MiniMacs columns as described by

Papyannopoulou (Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69; Papayannopoulou, T. et al., 1993, *Blood*, 81:229). The purity of isolated CDC34+ cells ranges routinely from 80-95%. Analgous infection studies can be repeated with selected adenovirus types on CD34+/CD38- subsets.

To confirm productive infection purified CD34+ cells can be infected with selected (methylase-tagged) serotypes and analyze viral DNA replication. Cultures of purified human bone marrow CD34+ cells can be used for the transduction and integration studies as a model for HSCs.

It was recently demonstrated that HSC activity does exist in CD34-negative human bone marrow subsets (Bathia, M. et al., 1998, *Nature Medicine*, 4:1038-45; Osawa, M., et al., 1996, *Science*, 273:242-5; Goodell. M. et al., 1997, *Nature Medicine*, 3:1337-45: Zanjani, E. D. et al., 1998, *Exp. Hematology*, 26:353-60). $Lin^{-}CD34^{-}38^{-}$ cells can be tested in the retargeting and transduction studies in combination with repopulation assays in SCID-NOD mice.

<u>G. Cloning and insertion of the fiber gene.</u>

METHODS

<u>PCR-cloning of the corresponding fiber gene and insertion into Ad5 based shuttle plasmids instead of the endogenous AD5 fiber:</u>

One or several adenoviruses with tropism to CD34+ or other HSC containing population is selected for further studies described herein. The complete coding region for fiber varies between 1-2kb, depending on the virus type. The fiber encoding sequences can be obtained by PCR with Pfu polymerase from viral DNA isolated from purified particles of the selected virus types. The corresponding primers can be designed based on the fiber sequences available from the EMBL gene bank. The PCR products are cloned as PacI-BalI fragment into pCD4 (Figure 10), a shuttle vector for recombination of RecA+ E.

coli. In pCD4, the heterologous fiber gene is flanked on both sides with Ad5 sequences, which are homologous to regions directly adjacent to the fiber reading frame in Ad5. As an Ad5 (shuttle vector) derived template for recombination, pCD1, a pBHG 10 (Microbix, Toronto, Canada) derivative can be used. The recombination procedure is performed according to a protocol routinely used for recombinant adenovirus generation (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Routinely, 90% of the resulting plasmids are accurately recombined. The junctions between the heterologous fiber (X) and Ad5 sequences can be sequenced to confirm the accuracy of recombination. The resulting plasmid is named pAd5fiberX ($pAd5^{fx}$). The resulting product is used to generate $pAd5^{fx}$-based Ad.AAV containing the heterologous fiber gene.

Construction of chimeric Ad vectors:

For transduction studies, two Ad vectors were constructed: Ad5GFP and Ad5GFP/F35, containing a chimeric Ad5/35 fiber gene. Both adenoviral vectors contained a 2.3kb, CMV promoter driven EGFP gene [derived from pEGFP-1, (Clontech, Palo Alto, CA)] inserted into the E3 region of Ad5. The EGFP expression cassette was cloned between Ad5 sequences 25,191-28,191 and 30,818-32,507 into a shuttle plasmid, which contained the E3 deletion described for pBHG10 (Microbix, Toronto, Canada). The resulting plasmid was named pAdGFP. For the chimeric vector, the Ad5 fiber gene in pAdGFP was substituted by an Ad5/35 chimeric fiber gene generated by the two-step PCR protocol outlined above. In the first PCR step, three DNA fragments corresponding to i) the Ad5 fiber 5'-nontranslated region and the first 132 bp of the fiber tail domain (nt 30,818-31,174), ii) the Ad35 shaft and knob domains (nt 132-991), and iii) the Ad5 E4 region including the Ad5 fiber polyadenylation signal (nt 32,775-33,651 were amplified by Pfu-Turbo DNA polymerase. The following primers were used: for the Ad5 tail, Ad5F-2 (nt 30,798-30,825) 5'-CGC GAT ATC GAT TGG ATC CAT TAA CTA-3' and Ad5R-2 (nt 31,174-31,153) 5'-CAG GGG GAC TCT CTT GAA ACC CAT T-3'; for the Ad35 shaft and knob, primers Ad5/35F and Ad5/35R (see above); for the Ad5E4 and polyA, primers Ad5F-1 and Ad5R-1 (see above). After 10 PCR cycles, the products were purified by agarose gel electrophoresis, combined, and then subjected to a second PCR

with primers Ad5F-2 and Ad5R-1. The resulting 2115 bp-long chimeric fiber gene contained the Ad5 tail and the Ad35 shaft and knob domains. This product was used as a substitute for the SalI/XbaI Ad5 fiber gene containing fragment in pAdGFP. The resulting plasmid was named pAdGFP/F35. To generate full-length E1/E3 vector genomes, pAdGFP and pAdGFP/F35 were inserted in pAdHM4 (Mizuguchi, H., Kay, M.A. 1998. *Human Gene Therapy*. 9:2577-2583) by recombination in *E.coli* (Chartier, C., E. et al. 1996. *Journal of Virology*. 70:4805-4810). To do this, the RecA+ *E.coli* strain BJ5183 was co-transformed with pAdHM4 linearized by SrfI mixed with the XbaI fragments containing the GFP genes, the Ad5 or Ad5/35 fiber genes, and the Ad5 homology regions. The resulting recombinants were analyzed by restriction analysis. Correct recombinants were amplified in *E.coli* HB101 and purified by double CsCl gradient banding. The plasmids were named pAd5GFP and pAd5GFP/F35. The correct structure of the Ad5/35 chimeric fiber gene was confirmed by endonuclease digestion and sequencing part of pAd5GFP/F35. To produce the corresponding viruses, pAd5GFP and pAd5GFP/F35 were digested with PacI to release the viral genomes and transfected onto 293 cells as described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaques developed 7 to 10 days post-transfection in overlayed cultures. Recombinant viruses were propagated in 293 cells and purified by standard methods described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960).

Hemagglutination assay:

Twenty-five microliters of serial dilutions of Ad5, Ad35, or chimeric Ad5GFP/F35 virions in McIlvaine-NaCl buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 [pH 7.2], diluted 1:50 with 0.87% NaCl) were loaded onto 96 well plates. To each dilution, 25 μl of a 1% suspension of monkey erythrocytes (in McIlvaine-NaCl buffer) was added. The sedimentation pattern was determined after incubation for 1 hour at 37°C. All tests were performed in quadruplicates in at least two independent experiments.

Southern blot:

Extraction of genomic DNA, labeling of DNA fragments and hybridization were performed as described earlier (Lieber, A., C.-Y. et al. 1996. *Journal of Virology.* 70:8944-8960).

RESULTS

Construction/Characterization of chimeric fiber:

Previously. it has been shown that exchanging the fiber knob was sufficient to alter the tropism of chimeric Ad vectors (Chillon, M., et al. 1999. *J. Virology.* 73:2537-2540; Krasnykh, V., et al. 1998. *J. Virology.* 72:1844-1852; Stevenson, S. C., et al. 1997. *J. Virology.* 71:4782-4790). As outlined above, the length of the fiber shaft may critically determine the entry strategy of a particular serotype. Therefore, we decided to replace not only the Ad5 fiber knob but also the shaft. The chimeric Ad5/35 fiber contained the Ad5 tail (amino acid: 1-44) necessary for interaction with the Ad5 penton base linked to 279 amino acids from Ad35 including the shaft with 7 β-sheets and the knob (Fig. 16A). The endogenous Ad5 fiber polyA signal was used to terminate transcription of the chimeric fiber RNA. The combination of the Ad5 capsid including the RGD motif containing penton base with a short-shafted fiber could be risky because the natural distance between the fiber knob and the RGD motifs was disturbed. The Ad5 fiber was substituted by the chimeric fiber sequences based on an E1/E3 deleted Ad vector. This vector carried a CMV promoter-GFP reporter gene cassette inserted into the E3 region. The corresponding chimeric virus (Ad5GFP/F35) was produced in 293 cells at a titer of $>2x10^{12}$ genomes per ml. For comparison, an E1/E3 deleted Ad vector containing the original Ad5 fiber gene and the GFP expression cassette was generated (Ad5GFP). The titer and the ratio of physical to infectious particles was similar between Ad5GFP and Ad5GFP/F35 indicating that the fiber modification did not significantly alter the stability and/or growth properties of the chimeric vector. The correctness of the fiber modification was confirmed by restriction analysis of the Ad5GFP/F35 viral genome followed by

Southern blot hybridization (Fig. 16B). direct sequencing of the fiber-coding region, and a functional test for hemagglutination (HA) of monkey erythrocytes. The agglutination of erythrocytes is fiber knob-mediated; it is known that Ad5 does not agglutinate monkey erythrocytes whereas Ad35 efficiently does (Pring-Akerblom, P., et al. 1998. *J. Virology*. 72:2297-2304). In HA tests, Ad5GFP/F35 agglutinated monkey erythrocytes with the same efficiency as Ad35 at dilutions of up to 1:512. In contrast. no hemagglutination was observed with equivalent Ad5 dilutions. This clearly confirmed the functional activity of the chimeric Ad5/35 fiber incorporated into Ad5 capsid.

Generation of chimeric adenoviral vectors (Ad.AAV^{fx}) with heterologous fiber molecules: Adenoviruses with chimeric Ad5-Ad3 fiber are viable and can be produced at high titers (Krasnykh, V., et al., 1996. *J. of Virology*, 70, 6839-6846; Stevenson, S. C. et al., 1997, *J. Virology*, 71:4782-90). In order to test whether the fiber substitution described herein affects production or stability of adenoviruses, two E1-deleted first-generation, adenoviral vectors are produced with the AAV-□gal cassette in 293 cells using standard protocols. The vector is generated by recombination of pAd.AAV-BG (Fig. 17) with pCD1 (containing the endogenous Ad5 fiber); the other vector (with heterologous fiber) is the recombination product of pAd.AAV βGal and pAd5fiberX (pAd5^{fx}). Virus from single plaques is amplified on 293 cells. The production yield per 293 cell can be determined by plaque-titering of 293 cell Lysates. It is anticipated that the fiber modification will not critically affect the stability of chimeric vectors. Finally, bone marrow cells can be infected with the retargeted vectors. Two days after infection, live-cell cytometry is performed for β-gal expression using as substrate Fluorescein di-□-D-Galactopyranoside (FDG) (Cantwell, M.J. et al., 1996 *Blood* 88, 4676-4683; Neering, S. et al., 1996, *Blood*, 88:1147-55; Fiering, S. N. et al., 1991, *Cytometry*, 12:291; Mohler, W. et al., 1996, *PNAS*, 93:57) and the infected cells are characterized for morphology and surface markers. Before and during infection, bone marrow cells can be cultured in IMDM/FCS supplemented with thrombopoietin (Tpo), which supports the survival of HSC (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). Alternatively, retargeted vectors can be

generated with the AAV-GFP (green fluorescence protein) cassette and perform FACS analysis on transduced cells based on GFP and surface marker expression.

H. Competition studies of chimeric fiber protein Ad5/35.

Competition studies:

Cross-competition studies between Ad5. 35, and Ad5GFP/F35 (Fig. 18) for binding and internalization were performed in order to investigate in more detail the pathways which are used by the chimeric vector to infect target cells. Wild-type Ad35 and the chimeric vector Ad5GFP/F35 could recognize the same primary receptor as they competed with each other for the attachment to K562 cells (Fig. 19A, upper panel). This primary receptor is different from that used by Ad5, since neither Ad5 viral particles nor anti-CAR monoclonal antibodies (Fig. 19B, upper panel) were able to abrogate Ad35 or Ad5GFP/F35 binding. In competition studies for internalization, Ad35 and Ad5GFP/F35 competed with each other with equal efficiency. Ad5 and anti-α_v-integrin monoclonal antibodies (L230) (Figs. 19C, D; lower panel) did not inhibit internalization of Ad35 or the chimeric virus. To consolidate this data, K562 cells were infected with Ad5GFP and Ad5GFP/F35 after prior incubation of cells with anti-CAR or anti-α_v-integrins monoclonal antibodies followed by analysis of GFP-expressing cells. The transduction data mirror the results obtained in adsorption/internalization studies. In summary, this demonstrated that Ad35 and Ad5GFP/F35 use a CAR and α_v-integrin-independent pathway for infection of K562 cells; the structural elements which account for these specific properties are located within the Ad35 fiber and can be transplanted into Ad5 by fiber substitution.

Ad3 can efficiently interact with K562 cells (Fig. 12), although Ad3 and Ad35 belong to the same subgroup (B), the homology between amino acid sequences of their fibers is only about 60%. Therefore, we decided to test whether Ad3 could compete with Ad35 and Ad5GFP/F35 for attachment and internalization (Fig. 20). These studies demonstrated that Ad35 binding was not inhibited by Ad3 indicating the use of different

receptors. Interestingly, Ad3 slightly inhibited attachment of Ad5GFP/F35 (Fig. 20A, left panel). In addition to binding to the receptor common for the Ad35 and Ad5GFP/F35 fiber, the chimeric capsid (e.g. the Ad5 penton RGD motifs) may also interacts with a second cellular receptor that overlaps with elements involved in Ad3 binding. In cross-competition for internalization, pre-incubation of cells at 37°C with Ad35 and with chimeric virus significantly decreased internalization of [^{3}H]-labeled Ad3 (Fig. 20D, right panel). In the reverse experiment, Ad3 as competitor decreased the level of internalization by 30% for both, Ad35 and the chimeric virus (Fig. 20B, right panel). As expected, Ad5 and Ad3 did not compete for adsorption or internalization. As shown before (Fig. 19B), anti-CAR and anti-α_v-integrin antibodies did not block Ad3 interaction with K652 cells. In summary, we concluded that Ad35 and Ad5GFP/F35 bind to receptor/s different from that of Ad3, although they can use common structural elements for internalization, which are different from α_v-integrins.

Infection studies with chimeric virus:

It is established that Ad5GFP/F35 infected K562 cells by a CAR and α_v-independent pathway. It is possible that this property allows for efficient transduction of non-cycling CD34+ cells, which express scarcely CAR and α_v-integrins. To test this, the transduction properties of Ad5GFP and Ad5GFP/F35 vectors were analyzed on CD34+ cells, K562, and HeLa cells. Fig. 21 shows the percentage of transduced, GFP expressing cells depending on the MOI used for infection. Nearly 100% of HeLa cells were transduced with Ad5GFP and Ad5GFP/F35 at MOIs of >/=25. More than 95% of the K562 cells were transduced with Ad5GFP/F35 at MOIs of >/= 100, whereas the transduction rate was significantly lower with Ad5 where it increased with the MOI reaching a plateau at ~70% GFP-positive cells after infection with an MOI of 400. Transduction of CD34+ cells was about three fold more efficient with Ad5GFP/F35 than with Ad5GFP at all MOIs analyzed. Interestingly, at higher MOIs, the transduction rate did not rise proportionally with the viral dose and soon reached a plateau indicating that in both cases only specific subset/s of CD34+ cells were permissive to infection. In order to characterize in more detail these specific, permissive subset/s, additional transduction

studies were performed. First, the percentage of GFP expressing cells was determined in CD34+ fractions that were stained for α_v-integrins or CARs (Fig. 22). The low number of CAR positive CD34+ cells complicated accurate co-labeling studies, and there was no correlation between CAR expression and the proportion of transduced cells among CD34+ cells infected with Ad5GFP or Ad5GFP/F35. Interestingly, for Ad5GFP, 65% of all GFP expressing cells were positive for α_v-integrins, whereas less than 22% of GFP positive cells infected with the chimeric virus stained positive for □$_v$-integrin expression. While only 17% of the whole CD34+ population expressed GFP after Ad5GFP infection, the percentage of GFP–expressing cells in the CD34+/ □$_v$-integrins positive fraction was 50%. This indicates that Ad5GFP vector-mediated GFP expression was preferentially localized to α_v-integrin positive CD34+ subsets, whereas after infection with the Ad5GFP/F35 vector, GFP was expressed in a broader spectrum of CD34+ cells with most of them being α_v-integrin-negative.

Next, transduced cells were simultaneously analyzed for GFP as well as for CD34 and CD117 markers. As mentioned before, only about 90% of all cells used in our analysis were positive for CD34 at the time of infection, hence the multiparameter analysis for CD34 and GFP. A population of CD34+ cells is extraordinarily heterogeneous in morphology and stem cell capacity. The subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (*Ikuta, K., Weissman, I.L. 1992* Proc. Natl. Acad. Sci. USA. *89:1502-1506*; *Simmons, P. J., et al. 1994*. Expl. Hematology. *22:157-165*). Fig. 23 summarizes the analyses of GFP expression in correlation with these specific stem cell markers. While 54% of cells infected with chimeric vector were positive for GFP and CD34+, only 25% of cells infected with Ad5GFP expressed the transgene and CD34+ marker (Fig. 23A, lower panel). More importantly, based on GFP expression, the chimeric virus transduced 80% of c-kit positive cells, whereas the Ad5-based vector transduced only 36% (Fig. 23A, middle panel). In an additional experiment, CD34+ cells were sorted for CD117 expression prior to infection with Ad5GFP or Ad5GFP/F35 and, 24 hours post-infection, GFP expression was analyzed in this specific fraction (Fig. 23B). This analysis revealed that the chimeric vectors transduced 4 fold more CD34+/CD117+ than the Ad5GFP vector.

In conclusion, these results demonstrated that the chimeric Ad5GFP/F35 vector was clearly superior to the Ad5GFP vector in targeting and transduction of CD34+ cells. Furthermore, the data suggest that the spectrum of CD34+ cell subsets permissive for Ad infection was significantly different for the chimeric vector than for the Ad5 vector.

Analysis of viral genomes within CD34+ cells infected with the Ad5 and chimeric vectors:

So far, the transduction rate of CD34+ cells was measured based on GFP expression after infection with Ad5GFP and Ad5GFP/F35. Considering the extraordinary heterogeneity of CD34+ cells in morphological and functional parameters, GFP may not be expressed in all cell types that were efficiently infected. Reasons for this include that the CMV promoter may not be active in all cell types or that the regulation of transgene expression could differ between subsets on a post-transcriptional or post-translational level. To test this, we quantified the number of intracellular (transduced) viral genomes within GFP positive and GFP negative fractions of CD34+ cells infected with Ad5GFP and Ad5GFP/F35. To do this, twenty-four hours after infection, CD34+ cells were sorted for GFP positive and GFP negative fractions, which were subsequently used to isolate genomic DNA together with transduced viral DNA. The number of viral genomes was determined by quantitative Southern blot as described for Fig. 15. Per GFP-positive CD34+ cell, about 270 copies of the Ad5GFP/F35 viral genome were detected. Interestingly, a remarkable 200 copies of the Ad5GFP/F35 viral genome were found per GFP-negative CD34+ cell (Fig. 24A and 25). This demonstrated that not all infected cells expressed GFP and implies that the actual transduction rate was higher than 54% (GFP-positive cells). We concluded that the CMV promoter was not active in all transduced CD34+ subsets. No Ad5GFP vector specific signal was detected within infected CD34+ (GFP positive or negative) fractions by Southern blot which had a detection limit of 14 viral genomes per cell. From this, we can conclude that the vector DNA concentration per transduced cell was at least 20 times higher for Ad5GFP/F35 than for Ad5GFP.

Ad5GFP DNA was only detectable in DNA samples from infected CD34+ cells by Southern blot after prior PCR amplification with vector specific primers (Fig. 24B and 25). This indicates that the replication deficient Ad5 vector is present but at a very low copy number, which may be limited by intracellular genome stability. Using the PCR-Southern detection method, Ad5 vector DNA was also detected in GFP negative cells, supporting that the CMV promoter may not have been the optimal choice for transduction studies. It is notable that studies by others on viral genome analyses after infection of CD34+ cells with Ad5 vectors were performed only after prior PCR amplification (Mitani, K., et al. 1994. *Human Gene Therapy*. 5:941-948; Neering, S. J., et al. 1996.. *Blood*. 88:1147-1155).

DISCUSSION

The chimeric Ad5GFP/F35 vector has binding and internalization properties similar to Ad35. Therefore, the fiber substitution was sufficient to swap cell tropism from Ad5 to Ad35. The Ad5GFP/F35 capsid chimera contained the short-shafted Ad35 fiber incorporated into an Ad5 capsid, instead of the naturally occurring long-shafted Ad5 fiber. During Ad5 infection, interaction between the penton base and intergrins is required to induce viral internalization. For this interaction, the length of fiber shaft and the precise spatial arrangement of knob and RGD motifs are critical for the virus entry strategy. The natural spatial arrangement is disturbed when short-shafted heterologous fibers are inserted into the Ad5 capsid. Interestingly, the Ad5/35 capsid chimera allows for efficient infection, suggesting that the protruding RGD motives in the Ad5 penton base do not affect the interaction with the primary Ad35 receptor. So far, most of the chimeric viruses were generated by substituting only the Ad5 knob while maintaining the long Ad5 fiber shaft (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540; Krasnykh, V. N., et al. 1996. *J. Virology*. 70:6839-6846; Stevenson, S. C., et al. 1995. *J. Virology*. 69:2850-2857; Stevenson, S. C., et al. 1997. *J. Virology*. 71:4782-4790). The exception was an Ad5/7 chimeric virus (Gall, J., et al. 1996. *J. Virology*. 70:2116-2123), where the whole Ad5 fiber was substituted by the short-shafted Ad7 fiber. However, similar to the parental Ad5, the Ad5/7 chimera still required α_v-integrins for infection.

This Ad5GFP/F35 chimera is the first demonstration that despite the presence of RGD motifs within the Ad5 penton, the chimeric virus uses cell entry pathways determined primarily by the receptor specificity of the short-shafted heterologous fiber. This does not exclude that interaction with a secondary receptor may increase binding affinity. The latter is supported by the observation that Ad35 and Ad5GFP/F35 slightly differed in their ability to compete with Ad5 or Ad3 for binding. It is possible that Ad5/35 attachment involves, in addition to the high affinity fiber binding, interaction between Ad5 capsid proteins (e.g. RGD motifs) and secondary receptor/s that overlap with those used by Ad3 and Ad5.

This data indicate that infection with Ad5-based vectors is restricted to a specific subset of CD34+ cells. The percentage of GFP expressing cells after Ad5GFP infection of CD34+ cells reached a plateau at MOIs higher than 100 indicating that only a limited fraction of CD34+ cells was permissive to Ad5. Also, strong replication of wild type Ad5 in infected CD34+ cells may be the result of preferential transduction of a specific subpopulation of CD34+ resulting in a expression of early viral genes at a level sufficient to initiate viral replication. The presence of a specific subpopulation of CD34+ cells permissive to Ad5-vector infection was suggested by others (Byk, T., et al. 1998. *Human Gene Therapy.* 9:2493-2502; Neering, S. J., et al. 1996.. *Blood.* 88:1147-1155). In the present report, we further characterized this subpopulation and demonstrated that Ad5-based vectors preferentially infected α_v-integrin positive CD34+ cells. Integrins (includingα_v) are thought to be important for homing and trafficking of transplanted hematopoietic cells, however little is known about the correlation between α_v-integrin expression and the differentiation status of hematopoietic cells (Papayannopoulou, T., Craddock, C. 1997. *Acta Haematol.* 97:97-104; Roy, V., Verfaillie, C.M. 1999. *Exp. Hematol.* 27:302-312). There was no clear correlation between CAR and GFP expression suggesting that Ad5GFP may be able to use another membrane protein as a primary receptor. Alternatively, Ad5GFP transduction observed at an MOI of 200-400 could be the result of direct interaction between virus and α_v-integrins triggering internalization, which may be the preferred pathway in the absence of CAR (Legrand, V., et al. 1999. *J.*

Virology. 73:907-919). Importantly, infection with the chimeric Ad5GFP/F35 vector was not restricted to the α_v-positive CD34+ subpopulation.

Among CD34+ cells, the subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). The receptor for stem cell factor, CD117 (c-kit) belongs to a tyrosine kinase family. It was previously shown that c-kit+, CD34+ cord blood cells contain a high fraction (16%) of hematopoietic progenitors (Neu, S., et al. 1996. *Leukemia Research*. 20:960-971). Early in ontogeny 34+/CD117+ cells have long-term repopulating activity (Sanchez, M. J., et al. 1996. *Immunity*. 5:513-525). An average of 50-60% of CD34+ cells are reported to be CD117 positive (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Neu, S., et al. 1996. *Leukemia Research*. 20:960-971; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). In our studies, the chimeric vector expressed GFP in 54% CD34+ cells and 80% of CD34+/c-kit+ cells. The actual viral transduction rate could be even higher because transduced Ad5GFP/F35 vector DNA was also found in GFP-negative fractions of infected cells. This indicates that the CMV promoter used to drive GFP expression in our vectors was not active in all transduced cells. We selected the CMV promoter for transgene expression based on published data demonstrating that PGK and CMV promoters allowed for efficient transgene expression in CD34 cells whereas the HTLV-I and RSV promoter were almost inactive (Byk, T., et al. 1998. *Human Gene Therapy*. 9:2493-2502; Case, S. S., et al. 1999. *Proc. Natl. Acad. Sci. USA*. 96:2988-2993). On the other hand, studies by Watanabe et al. (Watanabe, T., et al. 1996. *Blood*. 87:5032-5039) suggest that the CMV promoter is not active or rapidly silenced in certain CD34+ subsets. Our data underscore this observation. Considering retroviral transduction studies, the retroviral MLV promoter may have been a better candidate for transduction studies in hematopoietic cells (Bregni, M., et al. 1998. *Gene Therapy*. 5:465-472).

After having demonstrated that the Ad5GFP/F35 vector efficiently transduced cells carrying stem cell specific markers, the next logical step would be to perform colony

assays with pre-sorted GFP positive/negative cells. However, this assay is complicated by the fact that infection with first generation Ad vectors is cytotoxic and affects the formation and growth of progenitor colonies in MC-cultures (Mitani, K., et al. 1994. *Human Gene Therapy*. 5:941-948; Watanabe, T., et al. 1996. *Blood*. 87:5032-5039). This side effect is caused by the expression of Ad proteins within transduced cells (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960; Schiedner, G., et al. 1998. *Nature Genetics*. 18:180-183; Yang, Y., et al. 1994. *Proc. Natl. Acad. Sci. USA*. 91:4407-4411). Some of these proteins (e.g E4-orf4, pTP, or E3-11.6k) have pro-apoptotic activity (Langer, S. J., Schaak, J. 1996. *Virology*. 221:172-179; Lieber, A., et al. 1998. *J. Virology*. 72:9267-9277; Shtrichman, R., Kleinberger, T. 1998. *J. Virology*. 72:2975-2983; Tollefson, A. E., A et al. 1996 *J. Virology*. 70:2296-2306). Clearly, this would affect the outcome of transduction studies with Ad5GFP/F35, which allows for the efficient transfer of viral genomes into CD34+ cells implying significant expression of viral proteins. Moreover, recently published data indicate that short-term colony assay mostly measure mature progenitors and do not represent a rigorous test for transduction of potential stem cells.

A definitive demonstration that Ad5GFP/F35 based vectors can transduce HSC requires colony assays or preferably, repopulation assays in SCID-NOD mice. We can perform these studies with gutless vectors (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) and integrating □Ad.AAV vectors devoid of all viral genes (Lieber, A., et al. 1999. *J Virol* 73:9314-24) generated based on Ad5GFP/F35 chimeric capsids. Alternatively, gutless, retargeted vectors could be used to transiently express a retroviral receptor on CD34+ cells to increase their susceptibility to infection with retroviral vectors based on an approach that we have published earlier (Lieber, A., et al.. 1995. *Human Gene Therapy*. 6:5-11).

Our finding that Ad5GFP/F35 can efficiently transduce hematopoietic cells with potential stem cell capacity represents an important step towards stable gene transfer into HSCs and gene therapy of blood disorders. Furthermore, the virological aspects of this invention contribute to a better understanding of adenovirus cell interactions.

I: Retargeting of Ad5 based vectors with modified fibers carrying specific ligand peptides for HSC and other cell types

Another alternative to make Ad5-capsid-based vectors suitable for HSC gene therapy is to incorporate the coding sequence for HSC specific peptides into the H1 loop region of the Ad5 fiber gene. The modification of the H1-loop was successfully exercised by Krasnykh et al. with a 7 amino-acid long FLAG peptide (DYDDDDK). Using phage display peptide libraries (Pascqualini, R. et al., 1996, *Nature*, 380:364-66), Renata Pasqualini (La Jolla Cancer Research Center) reported recently, at the First Meeting of the American Society for Gene Therapy, the identification of small peptide ligands specific for bone marrow cells. The corresponding sequences encoding these peptides can be added to modify the H1 loop sequence employing site-directed mutagenesis. Optimally, the ligands should allow for the efficient internalization of adenoviral particles based on a CAR- and integrin independent pathway. Modified adenoviral vectors containing the AAVBG cassette can be produced and tested for HSC tropism as described above.

Adenovirus peptide display:

In order to retarget adenoviruses to any cell type of interest, a strategy is provided which involves creating a library of adenoviruses displaying random peptides in their fiber knobs as ligands and screening this library for adenovirus variants with tropism to a particular cell type in vitro and potentially in vivo.

The development of the adenovirus peptide display technique is based on the following ideas. (i) Although the tertiary structure of the Ad5 fiber knob is known, it remains unclear which domains are involved in receptor binding. There are data suggesting that receptor-binding domains partially overlap with hemagglutination domains, which are well characterized for a number of serotypes. Therefore, three intramolecular loop regions representing potential receptor binding sites can be substituted by random peptide

libraries. Eight amino acid residues in the center of the FG, or GH loops can be substituted by octameric random peptides (Figs. 26 and 27). These substitutions will replace CAR tropism and allow for infection of refractory cell types. (ii) To synthesize the oligonucleotides encoding the peptide library a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids is employed. This avoids termination codons and assures optimal codon usage and translation in human cells. Synthesis of a completely randomized library is possible with all 20 amino acids being incorporated with the same probability and a partially randomized library with only three (in average) random amino acids substitutions per octamer at random positions with a random amino acid to maintain certain critical features of the tertiary knob structure while introducing variability. The last model is based on the distribution of amino acids present in the hypervariable CDR 1 or 2 region of immunoglobulins. (iii) To maintain a representative library size of about 10^{10} different octamers per modified loop, a new cloning strategy is employed to allow for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. This strategy is based on a "seamless" cloning technique available from Stratagen. (iv) In order to produce the library of viruses, viral genomic DNA containing the modified fiber sequences is transfected into 293 cells without reduction of the library size. This critical step is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via poly-lysine to assure 100% transfection efficiency. This technique allows for coupling of ~1μg of plasmid DNA (or ~$1x10^{10}$ adenoviral genomes) to 10^{10} viral particles which can be used to infect 293/cre cells at an MOI of 10-100. Importantly, the carrier adenoviral genome has the packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express cre recombinase (293/cre). This helper virus system is routinely used to produce so-called gutless adenoviruses. Therefore, the virus progeny represents library genomes packaged into capsids containing preferentially Ad5 fibers. This is important for the next infection step into 293 cells at a MOI of 1 to assure a homogeneous fiber population on the capsid where the fibers are encoded by the packaged genome.

J. Production Of Adenovirus Vectors With Increased Tropism To Hepatocytes

An example of a G-H loop substitution to target Ad5 to hepacytes was successful. Preliminary tests demonstrated that two evolutionarily conserved regions within the malaria circumsporozoite surface protein (CS) termed RI and RII+ mediate specific interaction with hepatocytes but not with other organs (including spleen, lung, heart and brain), nor with Kupffer cells, liver endothelial cells or with other regions of the hepatocyte membrane (Cerami, C. et al., 1992, *Cell*, 70:1021-33; Shakibaei, M. and U. Frevert, 1996, *J. Exp. Med.*, 184:1699-711). These regions are conserved among different species including Plasmodium berghei, P. cynomogli, and P. falciparum that infect mouse, monkey and human hepatocytes, respectively (Cerami, C. et al., 1992, *Cell*, 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81). Peptides derived from RI (KLKQPG) or RII (EWSPCSVTCGNGIQVRIK) blocked CS binding to hepatocytes and infection by sporozoites in vivo ((Cerami, C. et al., 1992, *Cell*, 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81). RI and RII+ peptides were separately inserted into Ad5-fiber knob (H-I and G-H loop) containing mutation with abolished binding to CAR and alpha-v integrins (Kirby, L. et al., 2000, *J. Virol.*, 74:2804-13; Wickham, T. J. et al., 1995, *Gene Ther.*, 2:750-6). Based on preliminary data, a short-shafted fiber was used so that the virus entry strategy predominantly depends on the interaction with the primary (hepatocyte-specific receptor). The hepatocyte-specific ligands are flanked by short glycine stretches to provide flexibility and embedded into a loop formed by two cystines. This is one of the classical strategies to incorporate ligands into a protein scaffold (Doi, N. and H. Yanagawa, 1998, *Cell Mol. Life Sci.*, 54:394-404; Koivunen, E. et al., 1995, *Biotechnology (NY)*, 13:265-70) and to guarantee their presentation at the protein surface. The biodistribution of the best variants is tested in vivo in C57Bl/6 mice based on Southern blots or PCR for vector DNA in different organs. This mouse strain in known to be susceptible to infection with *P. berghei* (Chatterjee, S. et al., 1995, *Infect Immun.*, 63:4375-81).

K. Production Of Adenovirus Vectors With Increased Tropism To Tumor Cells

A similar strategy is to insert two peptides obtained after selection for tumor tropism by displaying random peptides on filamentous phages. The first double cyclic peptide (RGD-4) proved to bind specifically to integrins present on tumor vasculature (Ellerby H. M. et al., 1999, *Nat. Med.*, 5:1032-8). The second peptide targets specific matrix metalloproteinases associated with metastatic tumor cells as shown for the breast cancer cell line MDA-MB-435 (Koivunen, E. et al., 1999, *Nat. Biotechnol.*, 17:768-74). Tropism-modified vectors are tested in animal models with hepatic metastases derived from MDA-MB-435 cells (Fig. 28).

L. Development Of A Peptide Display Technique Based On Adenoviruses

A synthetic peptide library is described that allows adenovirus vectors to express random peptides in the G-H loop of the fiber knob domain. The technique of a phage display library is optimized to generate a library of adenoviruses displaying random peptides in their fiber knob. This library of adenovirus variants is then screened for tropism to a particular cell type in vitro and potentially in vivo. The oligonucleotides encoding the peptide library employ a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids. This will end the termination codons and assure optimal codon usage and translation in human cells. To maintain a representative library size, a new "seamless" cloning strategy that allows for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. Transfection into 293 cells is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via polylysine to assure a 100% transfection efficiency. Importantly, the carrier adenoviral genome has its packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express Cre recombinase (293/cre). The library is produced with E1-positive viruses depleted for CAR and integrin tropism. Only variant that have successfully infected the cell type of interest will replicate, resulting in de novo produced

virus. The sequence of the peptide ligand that conferred the particular tropism will then be analyzed in do novo produced virus.

EXAMPLE III

COMBINATION NOVEL ADENOVIRAL VECTOR AND MODIFIED FIBER PROTEIN

This example describes the following studies which combine the technology of the integrating adenovirus vector that is devoid of all adenoviral genes with the modified fiber protein that retargets the vector to quiescent HSC.

A. Transduction studies with re-targeted vectors in HSC:

In order to transduce quiescent HSC and integrate into chromosomal DNA, retargeted ΔAd.AAV^{fx} vectors are tested for reporter gene expression, and vector integration simultaneously while analyzing their clonogenic capacity. The modified ΔAd.AAV^{fx} hybrid vectors contain genomes devoid of all adenoviral genes (a "gutless" adenovirus vector) packaged into Ad5 capsids with modified fibers. Rep may be incorporated into these ΔAd.AAV^{fx} vectors to allow for site-specific integration into AAVS1.

Transduction studies:

Purified human CD34+ cells in IMDM/FCS+IL-3 and SCF are infected with different doses of ΔAd.AAV^{fx}-BG (1-10^7 genomes per cell). CD34+ cells infected with ΔAd.AAV^{fx}-βGal are cultured for 2 days in suspension and sort β-Gal+ cells by FACS using FDG as substrate. This determines the infection efficiency. β-gal expressing cells are then submitted to clonogenic assays in semi-solid cultures (in two dishes per MOI) in the presence of multiple cytokines. (IL-3, SCF, Epo, G-CSF, GM-CSF, IL-7, Tpo). A first set of semi-solid cultures can be evaluated after 7 days; another set can be analyzed after 14 days. Colonies that have formed in semisolid culture can be characterized by

light microscopy and subsequently stained with X-Gal staining. Most of the vector genomes should remain episomal and can be lost with successive cell divisions. Thus, while most cells can be X-Gal positive at day 2 or day 7 after infection, most of the larger colonies (analyzed at day 14 p.i.) may not stain homogeneously for β-Gal. A representative number of X-Gal positive and X-Gal negative colonies can be picked and analyzed for episomal and integrated vector DNA. The outcome depends on the MOI used for infection and the integration status of the vector. These studies determine whether hybrid vectors can infect primitive progenitors.

Detailed characterization of hybrid vector integration:

CD34+ cells can be infected with ΔAd.AAV^{fx}-SNori (MOI 1-10^7) and subjected to G418 selection in methyl cellulose (MC) cultures in the presence of growth factors (IL-3 and SCF). The resulting colonies are a mixture of mainly myeloid cells. The number and morphology of G418 resistant colonies can be determined after 2 weeks of selection. This strategy may be disadvantageous in that the appropriate stem cell may not divide and form G418 resistant colonies under the specific culture condition used. Moreover, it may be difficult to perform G418 selection on a population of heterogenous cells, which vary in their sensitivity to G418. Therefore, another set of ΔAd.AAV^{fx}-SNori infected CD34+ cells can be cultured in methyl-cellulose (+IL-3, SCF) without G418 selection. After 2-3 weeks, single colonies can be picked from both (w/ and w/o G418) MC cultures, morphologically characterized, and analyzed for integrated vector using the modified protocol developed for integration studies in a small number of cells (see Figure 8). This strategy allows the assessment of whether hybrid vectors integrate into the genome of CD34+ cells cultured in the presence of growth factors. This study gives us an idea about potential position effects affecting neo or βgal expression from integrated vector copies and about the structure of the integrated vector and the flanking chromosomal regions.

An alternative method to confirm vector integration:

Fluorescence *in situ* hybridization (FISH) analysis, can be performed in individual cells from MC colonies. CD34+ cells are cultured in MC in the presence of growth factors to induce cell division and subsequently treated with colchicine. Metaphase chromosome spreads are analyzed with biotin-ATP labeled probe specific for the βGal or SNori gene and a dioxigenin-UTP labeled probe for the human X-chromosome as an internal control (provided by Christine Disteche, University of Washington). Specific hybridization can be visualized with corresponding anti-biotin or anti-DIG antibodies labeled with different fluorochromes (e.g. FITC and Texas Red). Hybrid vector DNA may integrate as concatemers, which would facilitate detection by FISH. This technique allows one to localize the chromosomal integration sites of hybrid vectors.

Test transduction into quiescent bone marrow subpopulations:

Hybrid vectors described so far can be tested to see whether quiescent CD34+ cells can be stably transduced. To avoid significant cell proliferation, purified CD34+ cells are cultured in serum free IMDM supplemented with thrombopoietin (Tpo). Tpo can alone support the survival of stem cells without stimulating their active cell proliferation (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). To analyze the proliferation status of CD34+ cells at the time point of infection with the hybrid vector ΔAd.AAV^{fx}BG, BrdU is added 2 hours before infection to the culture medium. One set of cells are maintained as suspension culture in IDAM containing Tpo only for two days. Another set of cells are grown in IDAM+Tpo supplemented with multiple cytokines. Forty eight hours after infection, CD34+ cells can be FACS sorted for beta Gal expression using FDG. FDG positive cells can be further analyzed for cellular DNA replication based on BrdU incorporation and for specific CD34+ subset markers. To do this, cytospins from FDG+ cells can be submitted to immunofluorescence with BrdU specific antibodies and with antibodies to specific cell surface markers (e.g. CD38, CD41). Alternatively, consecutive paraffin sections of the same cell can be analyzed for (a) transgene expression by X-Gal staining, (b) DNA synthesis based on BrdU incorporation, and (c) specific surface markers. This allows one to confirm that the culture conditions with Tpo alone prevent

significant genomic DNA replication and subsequent cell proliferation as well as to determine whether quiescent CD34+ cells can be infected based on beta Gal expression in cells where BrdU labeling is absent.

Test hybrid vectors integration into quiescent CD34+ cells:

Two sets of CD34+ cells are infected. The first set of □Ad.AAV^{fx}SNori infected cells are cultured for 5-7 days in the presence of cytokines; the other set is cultured without cytokines. To maintain CD34+ cell viability without cytokines during this period, the cells are cultured in the presence of Tpo or underlaid with a stromal cell line (AFT024) (Moore, K. et al., 1997, *Blood*, 89:4337-47), which can maintain HSC viable for 4 to 7 weeks. After this specific time period, both sets are submitted to clonogenic assays (in the presence of multiple cytokines) either in combination with G418 selection or without selection. Single colonies are analyzed morphologically and submitted to genomic DNA analysis (Figure 8) to determine the vector integration status. The ultimate proof for stem cell transduction is the in vivo survival/expansion assay. To do this, the CD34+ cells expressing beta Gal are used for transplantation experiments, If the number of FDG+ cells is not sufficient, total ΔAd.AAV^{fx}BG infected cells as well as all ΔAd.AAV^{fx}-SNori infected cells can be used directly without selection. Transplantation can be performed via tail vein injection into sublethally irradiated SCID NOD mice (Dao, M.A., et al., 1998, *Blood,* 4, 1243-1255; Matsunaga, T. et al., 1998, *Blood*, 92:452-61). At different time points after transplantation (4 to 8 weeks), mice can be sacrificed to obtain bone marrow cells which then can be cultured in suspension until various assays are performed for X-Gal and cell markers as described earlier. These cells also can be submitted to a secondary colony assay in MC or secondary transplantation into SCID NOD mice. Furthermore, MC colonies derived from these cells can be analyzed for the presence of integrated vector DNA by the method illustrated in Figure 8. The expression and integration data together allow conclusions about the repopulation efficiency and about potential position effects.

B. Optimization of ΔAd.AAV^{fx} vectors for γ r-globin expression in hematopoietic stem cells:

One specific example of the invention is (a) to construct retargeted hybrid vector with the γ -globin as the transgene under the control of erythroid cell specific promoter, (b) to analyze the level and kinetics of γ -globin expression after transduction with hybrid vectors in *in vitro* and *in vivo* assays, (c) if required, to protect gene expression from position effects using γ -globin LCRs or insulators incorporated into hybrid vectors, and (d) to study whether γ -globin introns or heterologous introns can increase γ -globin expression.

Another central issue of the invention is to demonstrate that hybrid vectors can accommodate larger transgenes than rAAV and retroviruses. The insert size limitation of these vectors is 5kb. Transgene cassettes up to 8kb can be inserted into hybrid vectors as described. The maximal insert size may be about 14kb, if hybrid vectors are produced on the basis of E2a and/or E4 deleted rAd vectors in corresponding packaging cell lines. The maximal insert size in hybrid vectors is dictated by the packaging limit of first generation vectors (Ad.AAV) (<36kb) which are necessary intermediates for hybrid virus production at large scale. It is expected that stability and titer of Ad.AAV vectors with an 8kb globin gene cassette is comparable to the vector containing the 2.5-3.5kb cassette used in Ad.AAVBG, Ad.AAV1, and Ad.AAVSNori. The following example experiments address these issues.

Production of ΔAd.AAV^{fx} with large globin expression cassettes:

In order to improve the condition of sickle cell disease, the expression level of the transferred γ-lobin gene must be at least 50% of that of each endogenous βgene. These levels of transgene expression can only be achieved by using optimal expression cassettes, including extended LCRs and intron containing gamma genes. (Forrester, W.C., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 1359-1363; Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in*

Hematology, 35, 105-111; Martin, D. et al., 1996, *Current Opinion in Genetics and Development*, 6:488-95), So far, most of the γ-globin expression cassettes are designed for retroviral and rAAV vectors, thus, less than 5kb and have to be devoid of internal splice sites or poly adenylation signals. With integrating vectors described herein, it is possible to go beyond this size limitation. This allows one to improve γ -globin expression in bone marrow cells in terms of an adequate expression level and long term persistence. For this purpose, γ -globin constructs developed by Li et al (Emery, D. W., et al. 1999 *Hum Gene Ther* 10:877-88; Li, Q., et al. 1999. *Blood* 93:2208-16) or by Ellis et al (Ellis, J., et al., 1996, *EMBO J.*, 15. 562-568; Ellis, J., et al., 1997, *Nucleic Acids Res.* 25, 1296-1302) is chosen.

(i) The first cassette contains a γ -globin expression unit used in retroviral vectors. This allows for a direct comparison between the two systems. This construct includes the beta promoter from -127 to the beta initiation codon, which is connected in frame with the gamma coding region. This beta promoter is combined with the 300bp HS40 derived from the human alpha globin locus, which acts as a strong enhancer for globin expression. The globin gene is the 1.1kb version with intron 1 and partially deleted intron 2. A second cassette is generated containing the HS40 beta promoter and gamma globin gene with the complete 3.3kb gamma globin gene.

(ii) The second construct contains the 6.5kb beta μLCR, which confer a dominant chromatin opening activity and an adequate level of gamma globin expression in transgenic mice. The LCR is linked to the short 1.1kb version of the gamma globin gene or the complete 3.3kb gamma gene.

(iii) Additional globin expression cassette can be generated which include insulators, MARs or SARs, as well as other elements that can improve transgene expression from integrated vectors or in transgenic animals, like introns derived from the HPRT or hGH genes (Chung, J.H., et al., 1997, *Proc. Natl. Acad. Sci. USA* 94, 575-580; Dunaway, M., et al, 1993, *Mol. Cell. Biol.*, 17, 182-189; Felsenfeld, G., et al., 1996,

Proc. Natl. Acad. Sci. USA 93, 93840-9388; Klehr, D., et al., 1991, *Biochemistry*, 30, 1264-1270).

Transduction studies with ΔAd.AAVfx-globin vectors:

Transduction studies with globin-hybrid vectors are performed as described earlier (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13). Transduced CD34+ cells are submitted to differentiation in colony assays or analyzed *in vivo* expansion assays in SCID-NOD mice. MC-colonies or bone marrow cells from experimental mice are analyzed for globin expression. Gamma-globin expression is measured using fluorescent anti-gamma-globin antibodies. RNAase protection studies can be performed to specifically quantitate gamma globin mRNA in comparison with.-globin RNA. For these studies around 10^4-10^5 cells are needed per test.

Position effects:

In the absence of the LCR, globin genes are subjected to strong position effects when they are transferred into cultured CD34+ cells or erythroleukemic lines (Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111). Another concern is that site-specific integration of ΔAd.AAV/rep vectors into AAVS1 may silence transgene expression. If silencing happens, it can be overcome by incorporating LCRs such as the 6.5kb □ globin μLCR (Ellis, J., et al., 1996, *EMBO J.*, 15, 562-568; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111) or insulators into □Ad.AAV based expression units. Insulators are DNA elements that protect an integrated reporter gene from chromosomal position effects or that block enhancer activated transcription from a downward promoter. Insulator elements are known for Drosophila melanogaster genes (Gypsy, suppressor of Hairy wing, scs, scs', Fab-7). for the chicken beta-globin gene (HS4) and for the T cell receptor (BEAD1; 14, 21.25). Specifically, the Drosophila gypsy or the beta globin insulator can be inserted as two copies flanking the globin expression cassette into hybrid vectors. The position effects can be examined in transduced MC-colonies based on the

analysis of integrated vector DNA (see Fig. 29) and gamma-globin mRNA quantification. Analogous studies can be performed on transduced human bone marrow cells obtained after transplantation of infected CD34+ cells into SCID-NOD mice.

Intron effects on gamma-globin expression:

A number of reports reveal that the deletion of globin introns, particularly the second intron of the beta and gamma genes, decrease globin mRNA stability and thus the expression level (Antoniou, M. et al., 1998, *Nucleic Acid Res.*, 26:721-9). RNA viruses such as onco-retro, lenti- and foami viruses are problematic as vehicles for intron-containing transgenes. Because ΔAd.AAV is a DNA virus, it should package globin introns and LCRs if necessary without the decreased titers and rearrangements observed with retroviral vectors.

APPENDIX I

HUMAN AND ANIMAL ADENOVIRUSES AVAILABLE FROM AMERICAN TYPE CULTURE COLLECTION

1: Adenovirus Type 21 ATCC VR-1099 (NIAID V-221-002-014)

2: SA18 (Simian adenovirus 18) ATCC VR-943 Classification

3: SA17 (Simian adenovirus 17) ATCC VR-942 Classification

4: Adenovirus Type 47 ATCC VR-1309 Classification: Adenov

5: Adenovirus Type 44 ATCC VR-1306 Classification: Adenov

6: Avian adenovirus Type 4 ATCC VR-829 Classification: Ad

7: Avian adenovirus Type 5 ATCC VR-830 Classification: Ad

8: Avian adenovirus Type 7 ATCC VR-832 Classification: Ad

9: Avian adenovirus Type 8 ATCC VR-833 Classification: Ad

10: Avian adenovirus Type 9 ATCC VR-834 Classification: Ad

11: Avian adenovirus Type 10 ATCC VR-835 Classification: A

12: Avian adenovirus Type 2 ATCC VR-827 Classification: Ad

13: Adenovirus Type 45 ATCC VR-1307 Classification: Adenov

14: Adenovirus Type 38 ATCC VR-988 Permit: PHS permit requ

15: Adenovirus Type 46 ATCC VR-1308 Classification: Adenov

16: Simian adenovirus ATCC VR-541 Classification: Adenovir

17: SA7 (Simian adenovirus 16) ATCC VR-941 Classification:

18: Frog adenovirus (FAV-1) ATCC VR-896 Classification: Ad

19: Adenovirus type 48 (candidate) ATCC VR-1406 Classifica

20: Adenovirus Type 42 ATCC VR-1304 Classification: Adenov

21: Adenovirus type 49 (candidate) ATCC VR-1407 Classifica

22: Adenovirus Type 43 ATCC VR-1305 Classification: Adenov

23: Avian adenovirus Type 6 ATCC VR-831 Permit: USDA permi

24: Avian adenovirus Type 3 (Inclusion body hepatitis virus)

25: Bovine adenovirus Type 3 ATCC VR-639 Classification: A

26: Bovine adenovirus Type 6 ATCC VR-642 Permit: USDA perm

27: Canine adenovirus ATCC VR-800 Classification: Adenovir

28: Bovine adenovirus Type 5 ATCC VR-641 Permit: USDA perm

29: Adenovirus Type 36 ATCC VR-913 Classification: Adenovi

30: Ovine adenovirus type 5 ATCC VR-1343 Classification: A

31: Adenovirus Type 29 ATCC VR-272 Classification: Adenovi

32: Swine adenovirus ATCC VR-359 Classification: Adenoviru

33: Bovine adenovirus Type 4 ATCC VR-640 Permit: USDA perm

34: Bovine adenovirus Type 8 ATCC VR-769 Permit: USDA perm

35: Bovine adenovirus Type 7 ATCC VR-768 Permit: USDA perm

36: Adeno-associated virus Type 2 (AAV-2H) ATCC VR-680 Cla

37: Adenovirus Type 4 ATCC VR-4 Classification: Adenovirus

38: Adeno-associated virus Type 3 (AAV-3H) ATCC VR-681 Cla

39: Peromyscus adenovirus ATCC VR-528 Classification: Aden

40: Adenovirus Type 15 ATCC VR-661 Classification: Adenovi

41: Adenovirus Type 20 ATCC VR-662 Classification: Adenovi

42: Chimpanzee adenovirus ATCC VR-593 Classification: Aden

43: Adenovirus Type 31 ATCC VR-357 Classification: Adenovi

44: Adenovirus Type 25 ATCC VR-223 Classification: Adenovi

45: Chimpanzee adenovirus ATCC VR-592 Classification: Aden

46: Chimpanzee adenovirus ATCC VR-591 Classification: Aden

47: Adenovirus Type 26 ATCC VR-224 Classification: Adenovi

48: Adenovirus Type 19 ATCC VR-254 Classification: Adenovi

49: Adenovirus Type 23 ATCC VR-258 Classification: Adenovi

50: Adenovirus Type 28 ATCC VR-226 Classification: Adenovi

51: Adenovirus Type 6 ATCC VR-6 Classification: Adenovirus

52: Adenovirus Type 2 Antiserum: ATCC VR-1079 AS/Rab (NIA

53: Adenovirus Type 6 ATCC VR-1083 (NIAID V-206-003-014)

54: Ovine adenovirus type 6 ATCC VR-1340 Classification: A

55: Adenovirus Type 3 ATCC VR-847 (Derived from NIAID V-20

56: Adenovirus Type 7 ATCC VR-7 Classification: Adenovirus

57: Adenovirus Type 39 ATCC VR-932 Classification: Adenovi

58: Adenovirus Type 3 ATCC VR-3 Classification: Adenovirus

59: Bovine adenovirus Type 1 ATCC VR-313 Classification: A

60: Adenovirus Type 14 ATCC VR-15 Classification: Adenovir

61: Adenovirus Type 1 ATCC VR-1078 (NIAID V-201-001-014)

62: Adenovirus Type 21 ATCC VR-256 Classification: Adenovi

63: Adenovirus Type 18 ATCC VR-1095 (NIAID V-218-003-014)

64: Baboon adenovirus ATCC VR-275 Classification: Adenovir

65: Adenovirus Type 10 ATCC VR-11 Classification: Adenovir

66: Adenovirus Type 33 ATCC VR-626 Classification: Adenovi

67: Adenovirus Type 34 ATCC VR-716 Classification: Adenovi

68: Adenovirus Type 15 ATCC VR-16 Classification: Adenovir

69: Adenovirus Type 22 ATCC VR-257 Classification: Adenovi

70: Adenovirus Type 24 ATCC VR-259 Classification: Adenovi

71: Adenovirus Type 17 ATCC VR-1094 (NIAID V-217-003-014)

72: Adenovirus Type 4 ATCC VR-1081 (NIAID V-204-003-014)

73: Adenovirus Type 16 ATCC VR-17 Classification: Adenovir

74: Adenovirus Type 17 ATCC VR-18 Classification: Adenovir

75: Adenovirus Type 16 ATCC VR-1093 (NIAID V-216-003-014)

76: Bovine adenovirus Type 2 ATCC VR-314 Classification: A

77: SV-30 ATCC VR-203 Classification: Adenovirus, Simian (

78: Adenovirus Type 32 ATCC VR-625 Classification: Adenovi

79: Adenovirus Type 20 ATCC VR-255 Classification: Adenovi

80: Adenovirus Type 13 ATCC VR-14 Classification: Adenovir

81: Adenovirus Type 14 ATCC VR-1091 (NIAID V-214-001-014)

82: Adenovirus Type 18 ATCC VR-19 Classification: Adenovir

83: SV-39 ATCC VR-353 Classification: Adenovirus, Simian (

84: Adenovirus Type 11 ATCC VR-849 (Derived from NIAID V-2

85: Duck adenovirus (Egg drop syndrome) ATCC VR-921 Penni

86: Adenovirus Type 1 ATCC VR-1 Classification: Adenovirus

87: Chimpanzee adenovirus ATCC VR-594 Classification: Aden

88: Adenovirus Type 15 ATCC VR-1092 (NIAID V-215-003-014)

89: Adenovirus Type 13 ATCC VR-1090 (NIAID V-213-003-014)

90: Adenovirus Type 8 ATCC VR-1368 (Derived from NIAID V-20

91: SV-31 ATCC VR-204 Classification: Adenovirus, Simian (

92: Adenovirus Type 9 ATCC VR-1086 (NIAID V-209-003-014)

93: Mouse adenovirus ATCC VR-550 Classification: Adenoviru

94: Adenovirus Type 9 ATCC VR-10 Classification: Adenoviru

95: Adenovirus Type 41 ATCC VR-930 Classification: Adenovi

96: CI ATCC VR-20 Classification: Adenovirus, Simian (Mast

97: Adenovirus Type 40 ATCC VR-931 Classification: Adenovi

98: Adenovirus Type 37 ATCC VR-929 Classification: Adenovi

99: Marble spleen disease virus (Hemorrhagic enteritis virus

100: Adenovirus Type 35 ATCC VR-718 Classification: Adenovi

101: SV-32 (M3) ATCC VR-205 Classification: Adenovirus, Sim

102: Adenovirus Type 28 ATCC VR-1106 (NIAID V-228-003-014)

103: Adenovirus Type 10 ATCC VR-1087 (NIAID V-210-003-014)

104: Adenovirus Type 20 ATCC VR-1097 (NIAID V-220-003-014)

105: Adenovirus Type 21 ATCC VR-1098 (NIAID V-221-011-014)

106: Adenovirus Type 25 ATCC VR-1103 (NIAID V-225-003-014)

107: Adenovirus Type 26 ATCC VR-1104 (NIAID V-226-003-014)

108: Adenovirus Type 31 ATCC VR-1109 (NIAID V-231-001-014)

109: Adenovirus Type 19 ATCC VR-1096 (NIAID V-219-002-014)

110: SV-36 ATCC VR-208 Classification: Adenovirus, Simian (

111: SV-38 ATCC VR-355 Classification: Adenovirus, Simian (

112: SV-25 (M8) ATCC VR-201 Classification: Adenovirus, Sim

113: SV-15 (M4) ATCC VR-197 Classification: Adenovirus, Sim

114: Adenovirus Type 22 ATCC VR-1100 (NIAID V-222-003-014)

115: SV-23 (M2) ATCC VR-200 Classification: Adenovirus, Sim

116: Adenovirus Type 11 ATCC VR-12 Classification: Adenovir

117: Adenovirus Type 24 ATCC VR-1102 (NIAID V-224-003-014)

118: Avian adenovirus Type 1 (Chicken Embryo Lethal Orphan; C

119: SV-11 (M5) ATCC VR-196 Classification: Adenovirus, Sim

120: Adenovirus Type 5 ATCC VR-5 Classification: Adenovirus

121: Adenovirus Type 23 ATCC VR-1101 (NIAID V-223-003-014)

122: SV-27 (M9) ATCC VR-202 Classification: Adenovirus, Sim

123: Avian adenovirus Type 2 (GAL) ATCC VR-280 Classificati

124: SV-1 (M1) ATCC VR-195 Classification: Adenovirus, Simi

125: SV-17 (M6) ATCC VR-198 Classification: Adenovirus, Sim

126: Adenovirus Type 29 ATCC VR-1107 (NIAID V-229-003-014)

127: Adenovirus Type 2 ATCC VR-846 Classification: Adenovir

128: SV-34 ATCC VR-207 Classification: Adenovirus, Simian (

129: SV-20 (M7) ATCC VR-199 Classification: Adenovirus, Sim

130: SV-37 ATCC VR-209 Classification: Adenovirus, Simian (

131: SV-33 (M10) ATCC VR-206 Classification: Adenovirus, Si

132: Avian adeno-associated virus ATCC VR-865 Classificatio

133: Adeno-associated (satellite) virus Type 4 ATCC VR-646

134: Adenovirus Type 30 ATCC VR-273 Classification: Adenovi

135: Adeno-associated (satellite) virus Type 1 ATCC VR-645

136: Infectious canine hepatitis (Rubarth's disease, Fox ence

137: Adenovirus Type 27 ATCC VR-1105 (NIAID V-227-003-014)

138: Adenovirus Type 12 ATCC VR-863 (Derived from NIAID V-2

139: Adeno-associated virus Type 2 (molecularly cloned) ATCC

140: Adenovirus Type 7a ATCC VR-848 (Derived from NIAID V-2

What is claimed:

1. A first generation recombinant adenovirus vector a portion of which integrates into a host cell genome, comprising:
   a) A left adenovirus inverted terminal repeat sequence;
   b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
   c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
   d) At least one adenoviral sequence which directs adenoviral replication; and
   e) A right adenoviral inverted terminal repeat sequence,

   wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

2. The adenovirus vector of claim 1, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype.

3. The adenovirus vector of claim 1, wherein the sequence which directs adenoviral replication comprises a sequence on the anti-parallel strand which encodes an adenoviral fiber protein including a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

4. The adenovirus vector of claim 3, wherein the sequence on the anti-sense strand which encodes the fiber tail is from the same serotype as the adenoviral inverted repeat sequence.

5. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:
   a) A left cassette inverted terminal repeat sequence;
   b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the polyadenylation sequence;

d) A promoter sequence 3' to the polyadenylation sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

6. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;

b) A promoter sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the promoter sequence;

d) A polyadenylation sequence 3' to the transgene sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

7. The adenovirus vector of claim 5 or 6, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

8. The adenovirus vector of claim 5 or 6, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

9. The adenovirus vector of claim 8, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

10. The adenovirus vector of claim 8, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

11. The adenovirus vector of claim 8, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

12. The transgene cassette of claim 5 or 6 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

13. The transgene cassette of claim 5 or 6, further comprising an insulator element.

14. The transgene cassette of claim 5 or 6, further comprising a bacterial origin of replication.

15. The adenoviral vector of claim 1, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

16. A first generation recombinant adenovirus vector which targets a host cell of interest and a portion of which integrates into the host cell genome so targeted, comprising two DNA strands, each strand being antiparallel to the other, the first strand comprising:

a) A left adenovirus inverted terminal repeat sequence;
b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
d) At least one adenoviral sequence which directs adenoviral replication; and
e) A right adenoviral inverted terminal repeat sequence,

wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome, and wherein the second strand comprises a sequence which encodes an adenoviral fiber protein that permits targeting of the vector into the host cell of interest.

17. The adenovirus vector of claim 16, wherein the adenoviral protein includes a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

18. The adenovirus vector of claim 16, wherein the left and right adenovirus inverted terminal repeat sequences and the packaging sequence are from the same adenoviral serotype.

19. The adenovirus vector of claim 17, wherein the fiber tail is from the same serotype as the left and right adenoviral inverted repeat sequences.

20. The adenovirus vector of claim 17, wherein the fiber shaft is from a different serotype as the left and right adenoviral inverted repeat sequences.

21. The adenovirus vector of claim 20, wherein the fiber shaft is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

22. The adenovirus vector of claim 17, wherein the fiber shaft comprises a shortened length.

23. The adenovirus vector of claim 17, wherein the fiber knob is from a different serotype as the left and right adenoviral inverted repeat sequences.

24. The adenovirus vector of claim 23, wherein the fiber knob is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

25. The adenovirus vector of claim 17, wherein the fiber knob is a modified fiber knob protein comprising the G-H loop replaced with a heterologous peptide ligand sequence which binds to at least one surface protein on the host cell of interest.

26. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;

b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the polyadenylation sequence;

d) A promoter sequence 3' to the polyadenylation sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

27. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;

b) A promoter sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the promoter sequence;

d) A polyadenylation sequence 3' to the transgene sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

27. The adenovirus vector of claim 26 or 27, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

28. The adenovirus vector of claim 26 or 27, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

29. The adenovirus vector of claim 24, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin

30. The adenovirus vector of claim 24, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

31. The adenovirus vector of claim 24, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

32. The transgene cassette of claim 26 or 27 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

33. The transgene cassette of claim 26 or 27, further comprising an insulator element.

34. The transgene cassette of claim 26 or 27, further comprising a bacterial origin of replication.

35. The adenoviral vector of claim 16, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

36. A recombinant gutless adenovirus vector a portion of which integrates into a host cell genome, comprising:
   a) A left adenovirus inverted terminal repeat sequence;
   b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
   c) A transgene cassette sequence 3' to the adenoviral packaging sequence; and
   d) A right adenoviral inverted terminal repeat sequence,

   wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

37. The adenovirus vector of claim 36, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype. *(this is the base vector)*

38. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;
b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;
c) A transgene sequence 3' to the polyadenylation sequence;
d) A promoter sequence 3' to the polyadenylation sequence; and
e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

39. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;
b) A promoter sequence 3' to the left inverted terminal repeat sequence;
c) A transgene sequence 3' to the promoter sequence;
d) A polyadenylation sequence 3' to the transgene sequence; and
e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

40. The adenovirus vector of claim 38 or 39, wherein the left and the right inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

41. The adenovirus vector of claim 38 or 39, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

42. The adenovirus vector of claim 41, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin

43. The adenovirus vector of claim 41, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentomycin.

44. The adenovirus vector of claim 41, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

45. The adenovirus vector of claim 38 or 39 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

46. The adenovirus vector of claim 38 or 39, further comprising an insulator element.

47. The adenovirus vector of claim 38 or 39, further comprising a bacterial origin of replication.

48. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 5' portion of a gene of interest.

49. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 3' portion of a gene of interest.

50. A method of producing a resolved gutless adenovirus vector in a suitable cell, said method comprising introducing a first and a second adenovirus vectors of claim 1 or 16 into the cell under suitable conditions so that the recombinant adenovirus vectors undergo homologous recombination thereby producing a resolved gutless adenovirus vector.

51. A resolved gutless adenovirus vector produced by the method of claim 50.

52. The method of claim 50, wherein the first adenovirus vector comprises a transgene cassette having a 5' portion of a gene of interest, and wherein the second adenovirus vector comprises a transgene cassette having a 3' portion of the gene of interest, and wherein a part of the 5' portion overlaps with a part of the 3' portion so that homologous recombination occurs.

53. A method of producing a resolved gutless recombinant Ad vector by homologous recombination in a suitable cell, said method comprising contacting two parental recombinant Ad vectors, each comprising a transgene cassette containing a portion of a selected transgene with a region of overlapping homology, so that the first and second parental recombinant Ad vectors undergo homologous recombination at the region of overlapping homology, resulting in a resolved recombinant gutless Ad vector having both portions of the selected transgene, and wherein the selected transgene is within a transgene cassette flanked by a pair of ITRs.

54. A resolved gutless adenovirus vector produced by the method of claim 53.

55. An adenovirus library comprising a plurality of adenovirus vectors expressing fiber proteins which are displayed and modified by random peptide insertions.

56. The library of claim 55, wherein said fiber protein so displayed comprises a random peptide substituted in the G-H loop of the fiber protein knob domain.

57. A screening method for targeting adenovirus vectors for gene therapy comprising contacting the adenovirus library of claim 55 with a plurality of cells so that the cells are transduced with the adenovirus vectors of the adenovirus library transduction occurs and detecting the cells so transduced.

58. The adenovirus vector of claim 5, 6, 26, 27, 38, or 39 further comprising a nucleotide sequence encoding a rep78 protein.

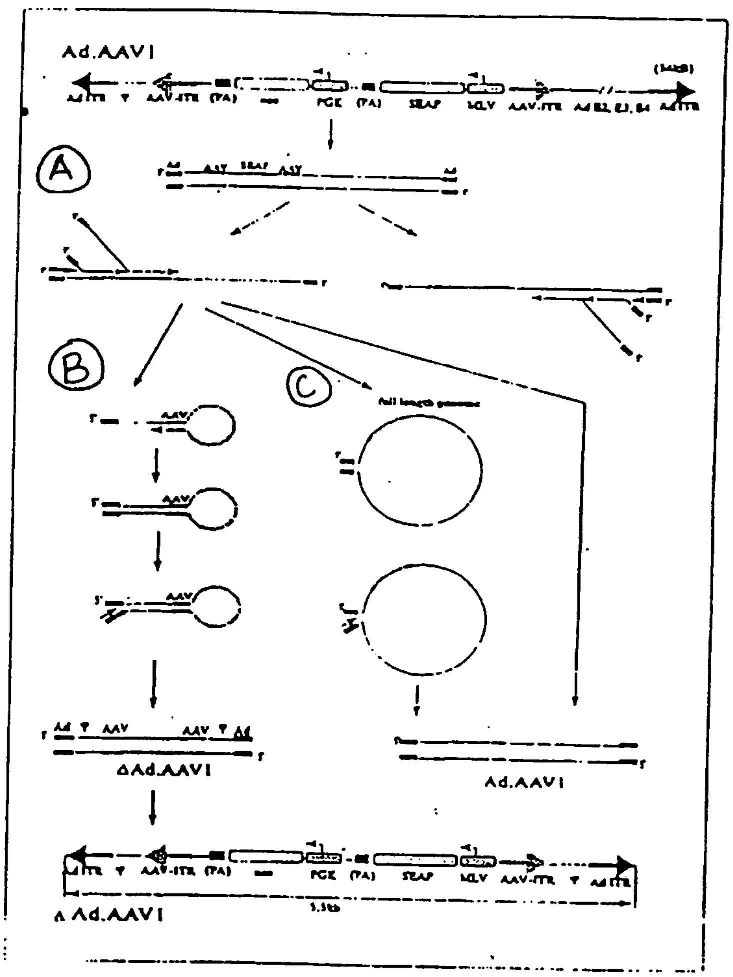


FIG. 1

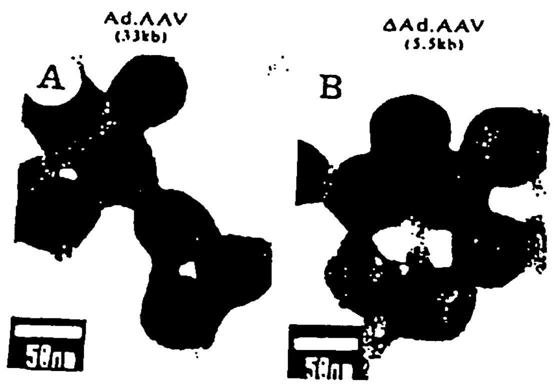


FIG. 2

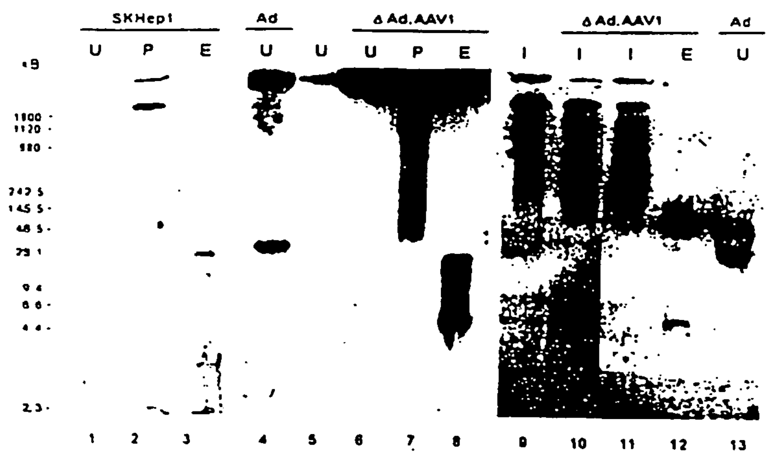
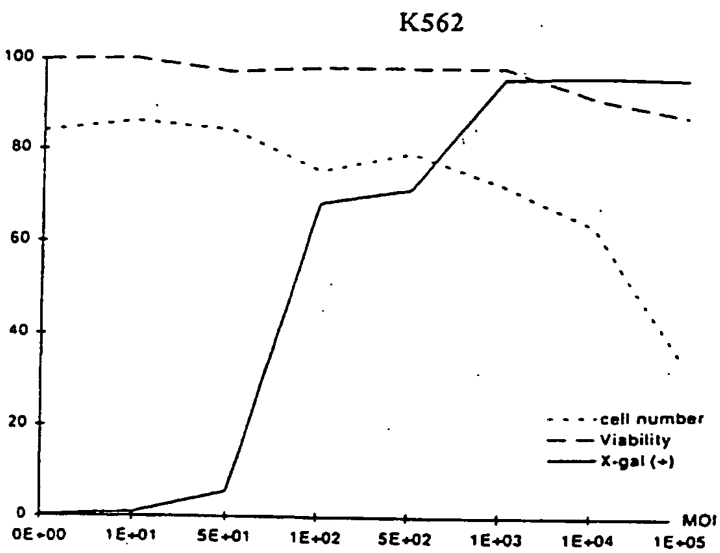
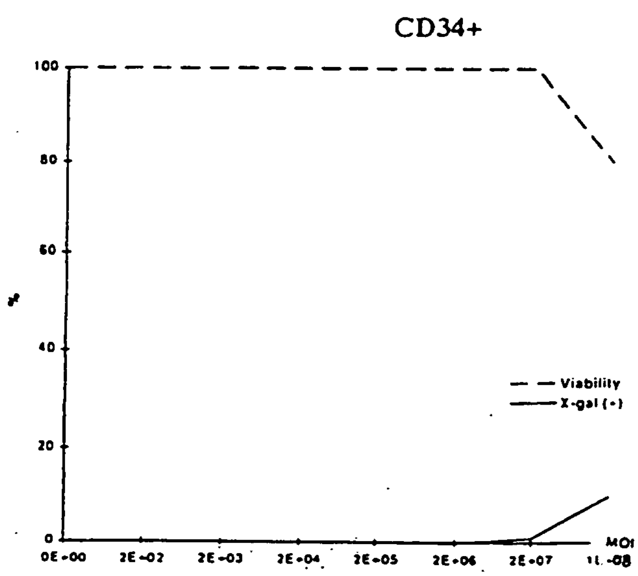

SKHep1
Ad
Δ Ad.AAV1
Δ Ad.AAV1
Ad
U P E U U U P E I I I E U
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 3



K562
100
80
60
40
20
0
0E+00
1E+01
5E+01
1E+02
5E+02
1E+03
1E+04
1E+05
MOI
cell number
Viability
X-gal (+)

FIG. 4A



CD34+
100
80
60
40
20
0
%
0E+00
2E-02
2E-03
2E+04
2E+05
2E+06
2E+07
MOI
Viability
X-gal (+)

FIG. 4B

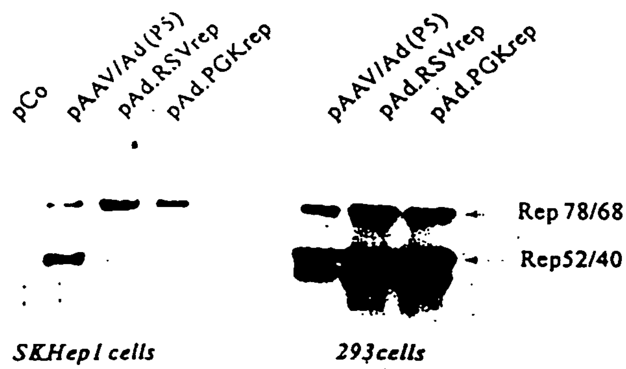

pCo
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
Rep 78/68
Rep52/40
SKHep1 cells
293cells

FIG. 5

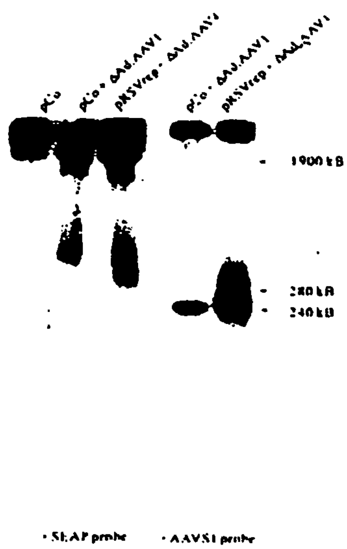

pCro
pCro + ΔAd.AAV1
pRSVrep + ΔAd.AAV1
pCro + ΔAd.AAV1
pRSVrep + ΔAd.AAV1
1900 kB
280 kB
240 kB
SEAP probe
AAVS1 probe

FIG. 6

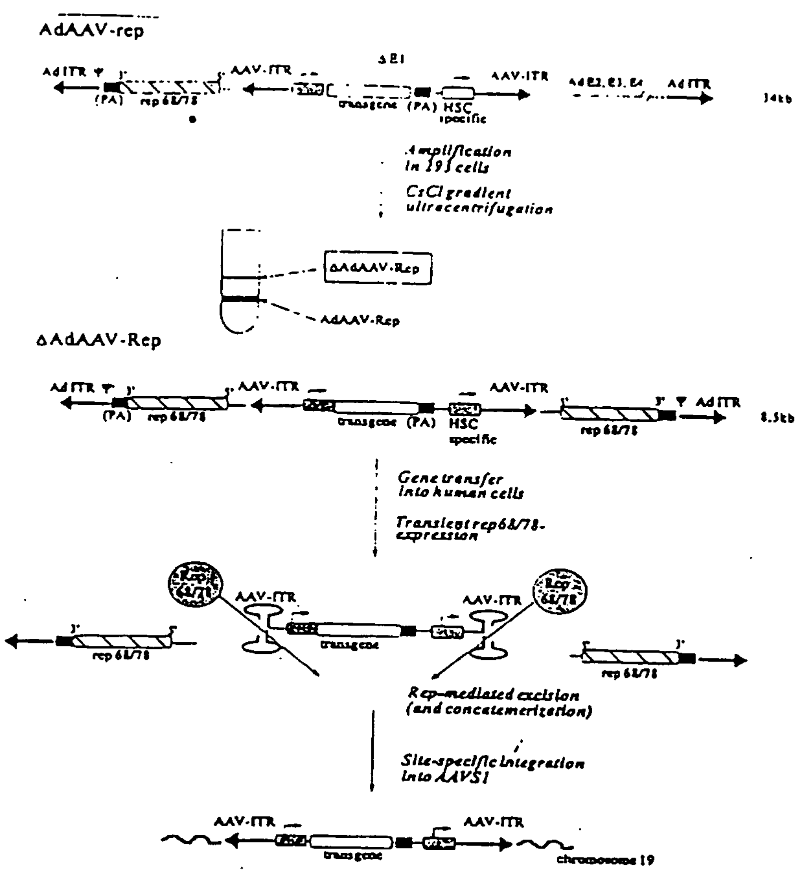
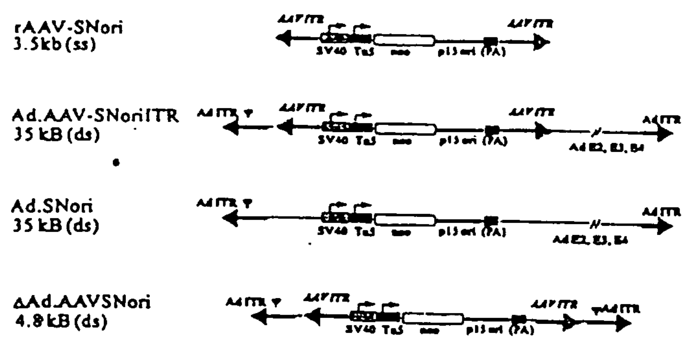
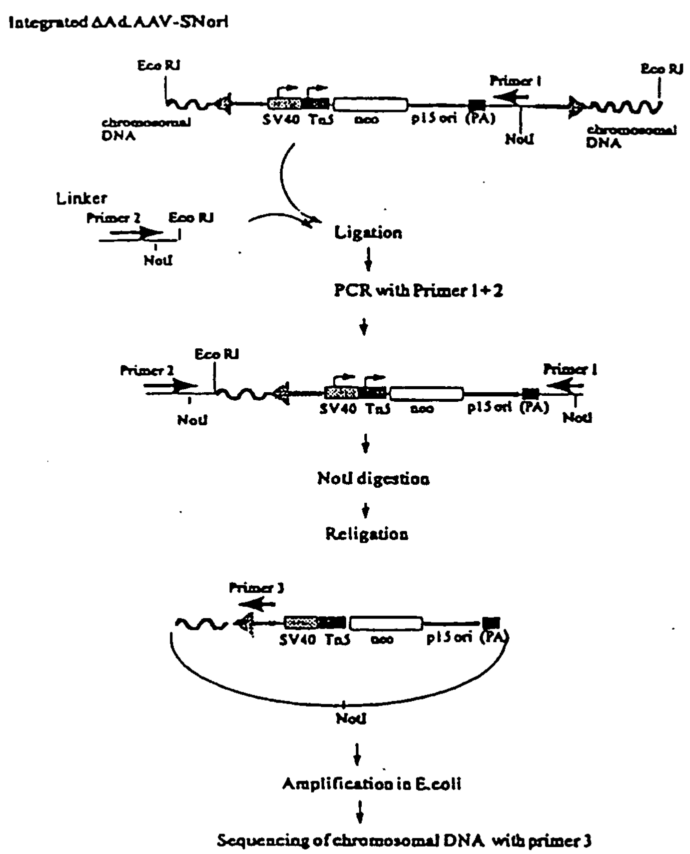

AdAAV-rep
AdITR
(PA)
rep 68/78
AAV-ITR
ΔE1
transgene
(PA)
HSC specific
AAV-ITR
AdE2, E3, E4
AdITR
34kb
Amplification in 293 cells
CsCl gradient ultracentrifugation
ΔAdAAV-Rep
AdAAV-Rep
ΔAdAAV-Rep
AdITR
(PA)
rep 68/78
AAV-ITR
transgene
(PA)
HSC specific
AAV-ITR
rep 68/78
AdITR
8.5kb
Gene transfer into human cells
Transient rep68/78-expression
Rep 68/78
AAV-ITR
transgene
AAV-ITR
Rep 68/78
rep 68/78
rep 68/78
Rep-mediated excision (and concatemerization)
Site-specific integration into AAVS1
AAV-ITR
transgene
AAV-ITR
chromosome 19

FIG. 7



rAAV-SNori
3.5kb (ss)
Ad.AAV-SNoriITR
35 kB (ds)
Ad.SNori
35 kB (ds)
ΔAd.AAVSNori
4.8 kB (ds)
SV40
neo
p15 ori
(PA)

FIG. 8A



Integrated ΔAd.AAV-SNori
Eco RI
Primer 1
chromosomal DNA
SV40
TnS
neo
p15 ori
(PA)
NotI
Linker
Primer 2
Ligation
PCR with Primer 1+2
NotI digestion
Religation
Primer 3
Amplification in E.coli
Sequencing of chromosomal DNA with primer 3

FIG. 8B

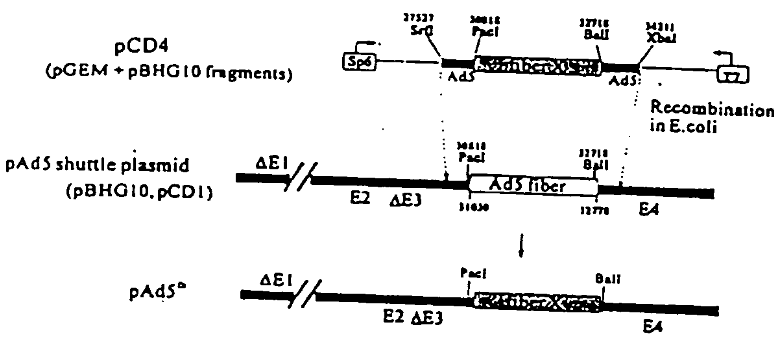

pCD4
(pGEM + pBHG10 fragments)
Sp6
SrfI
PacI
BalI
XbaI
T7
Ad5
Ad5
Recombination in E.coli
pAd5 shuttle plasmid
(pBHG10, pCD1)
ΔE1
PacI
BalI
Ad5 fiber
E2 ΔE3
E4
pAd5
ΔE1
PacI
BalI
E2 ΔE3
E4

FIG. 9

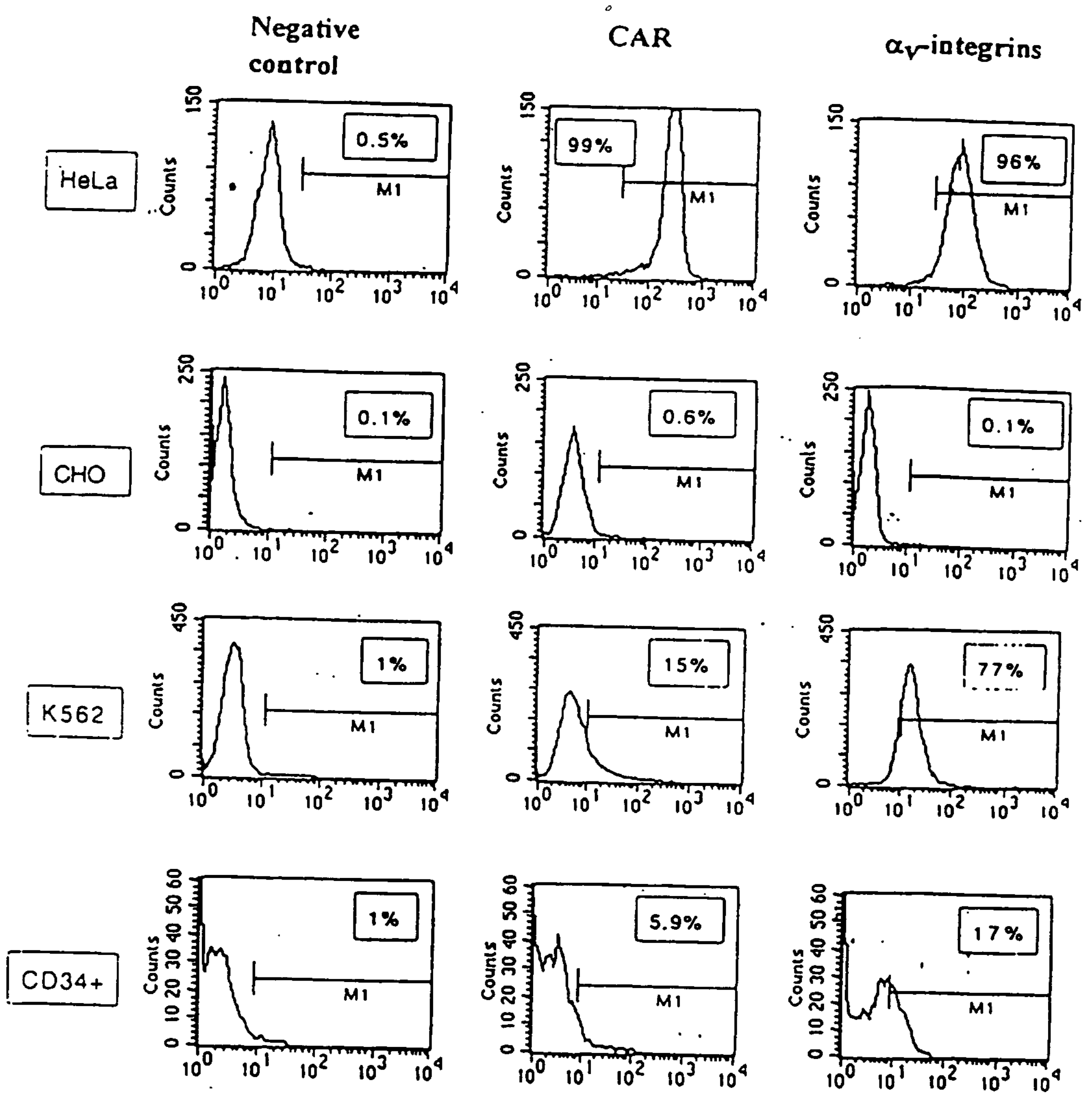
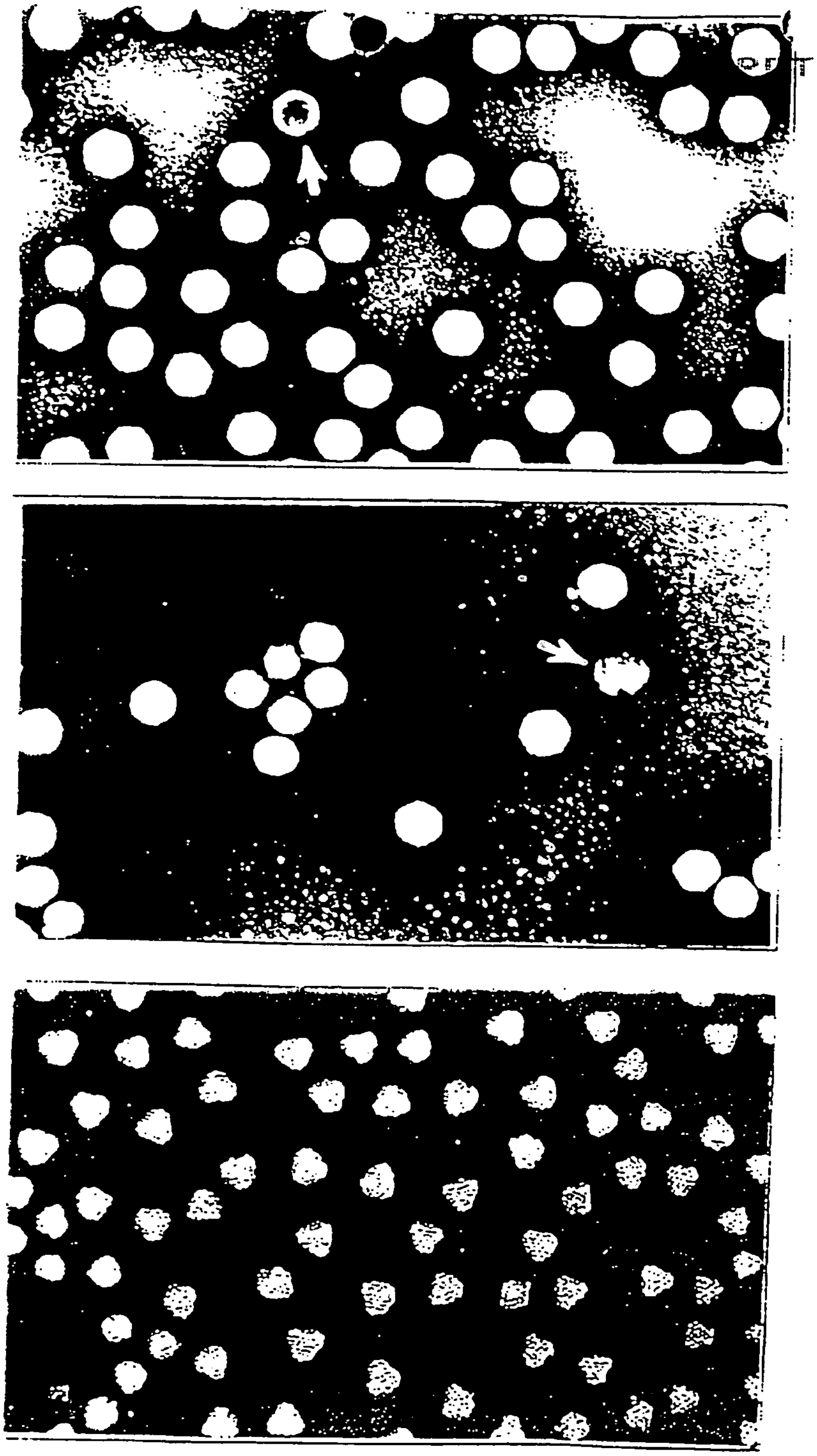

Negative control
CAR
α_V-integrins
HeLa
CHO
K562
CD34+
0.5%
99%
96%
0.1%
0.6%
0.1%
1%
15%
77%
1%
5.9%
17%
M1
Counts

FIG. 10



Ad35

Ad9

Ad5

Fig. 11

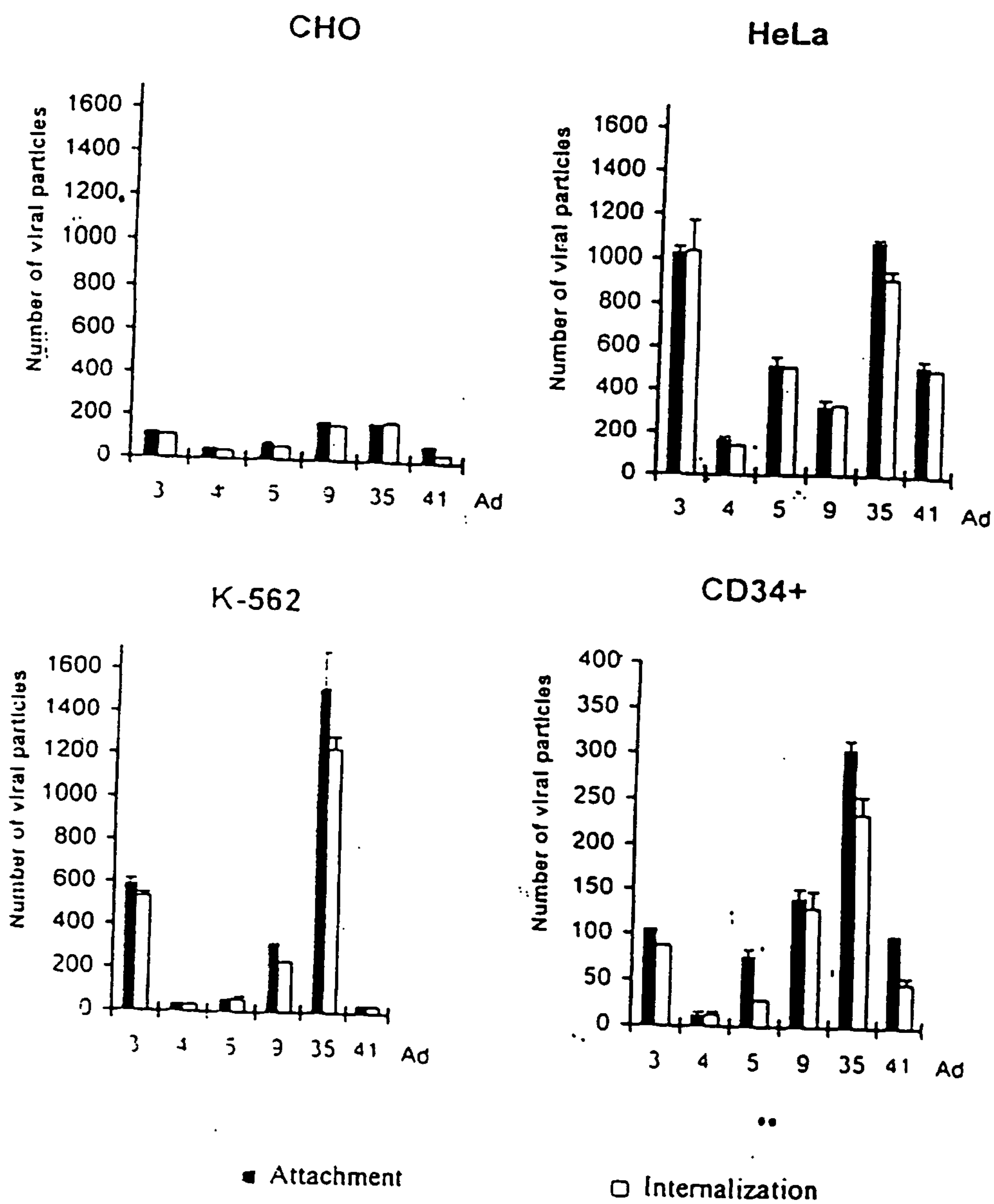

CHO
HeLa
K-562
CD34+
Number of viral particles
Ad
3
4
5
9
35
41
Attachment
Internalization

FIG. 12

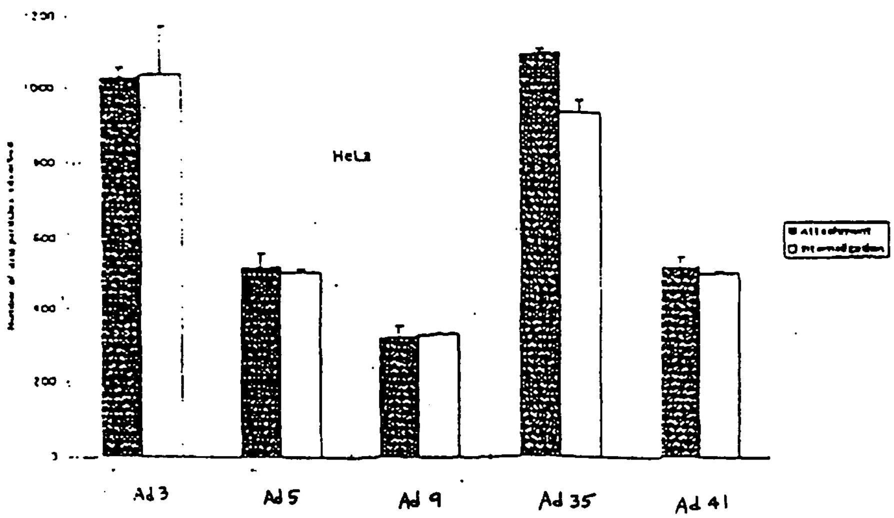


FIG. 13 A

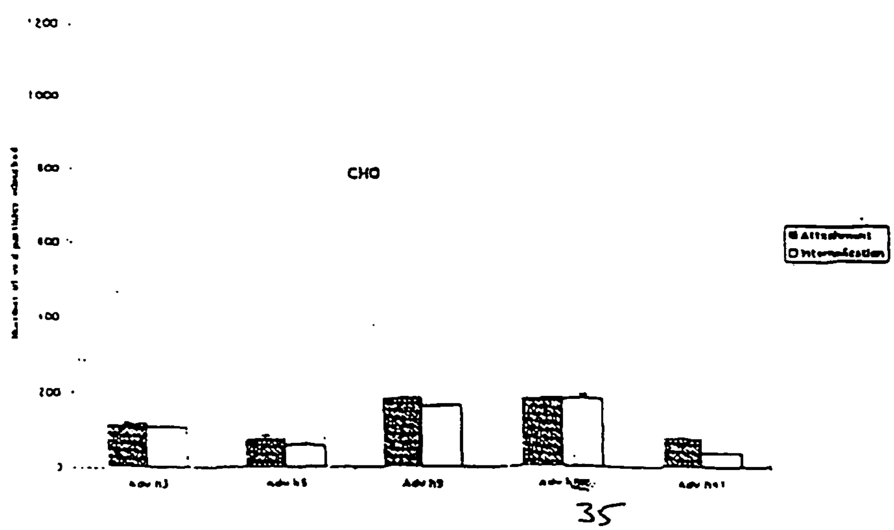


FIG. 13 B

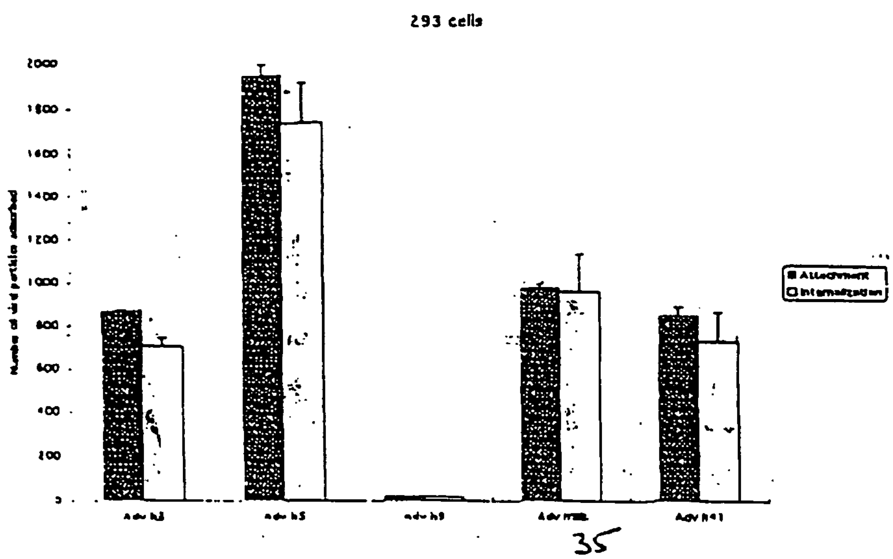

293 cells
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Attachment
Internalization
35

FIG. 13C

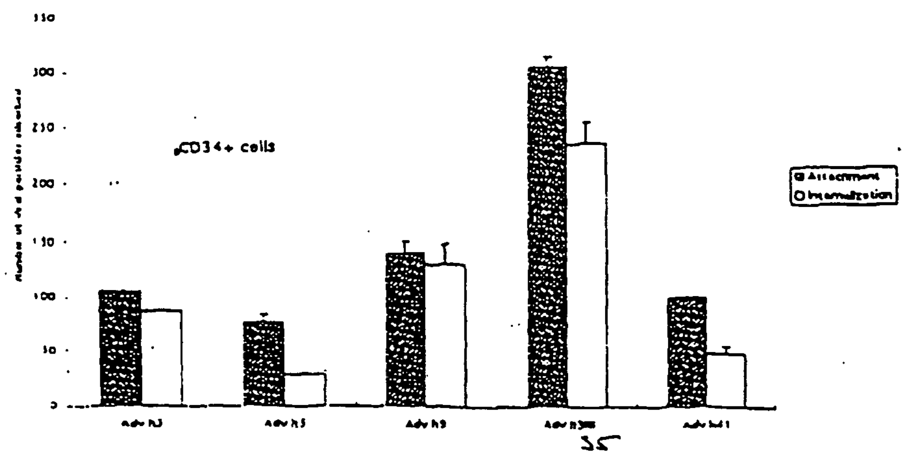


FIG 14A

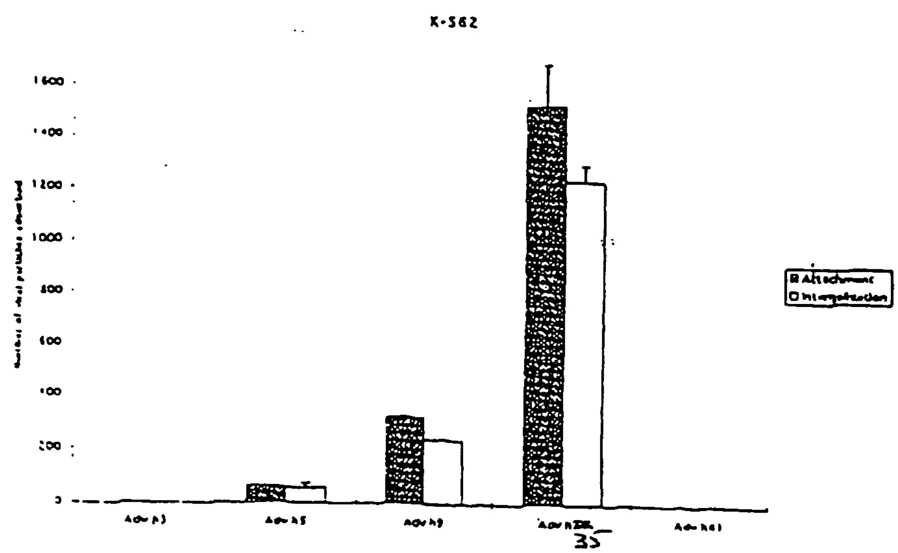


FIG 14B

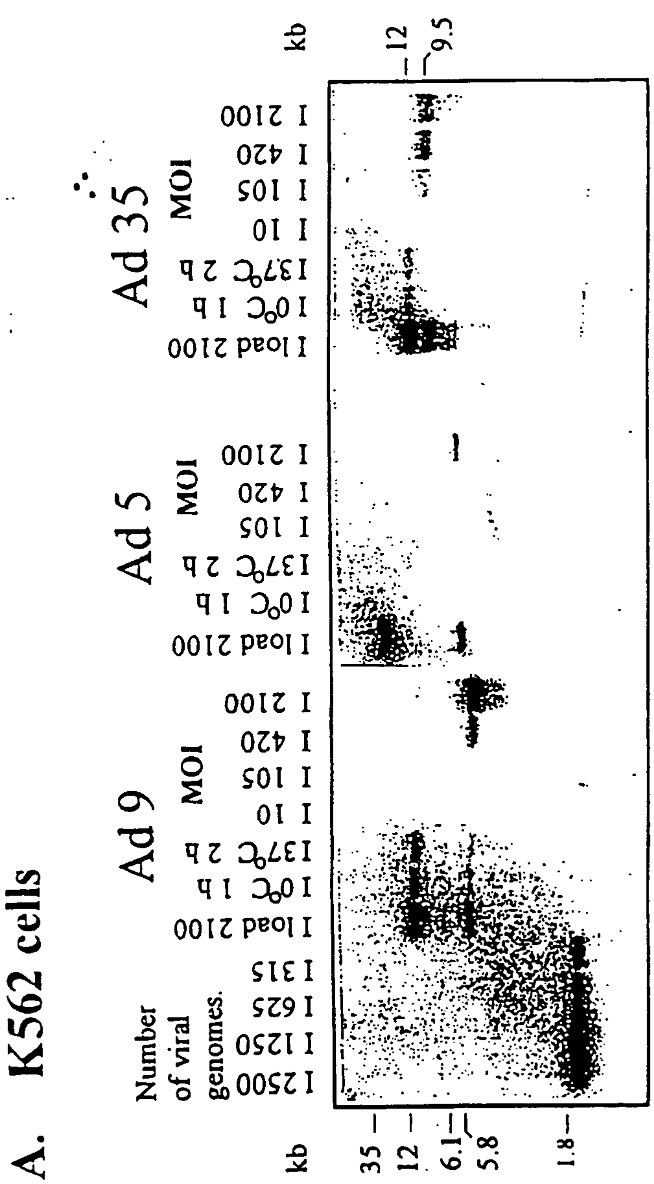
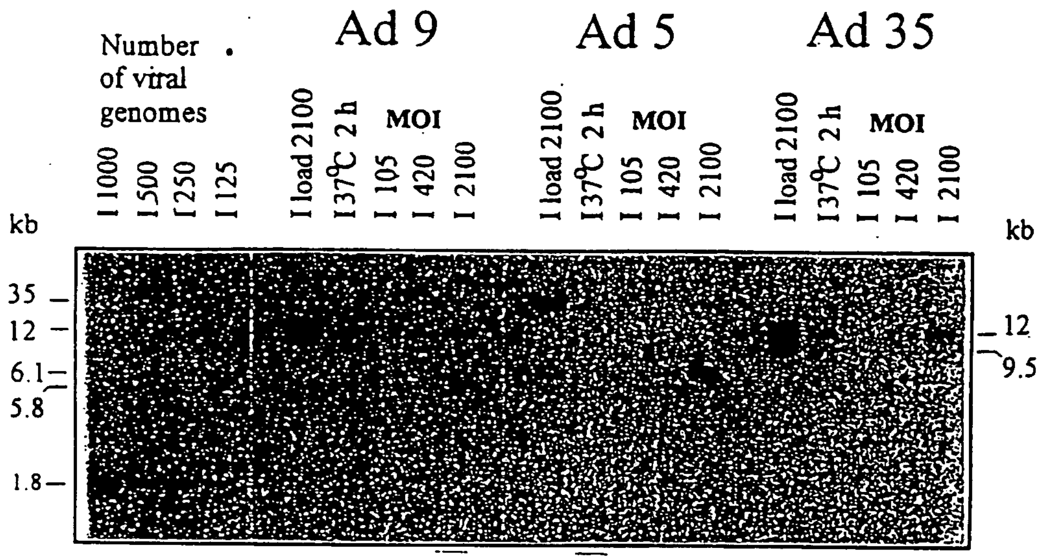
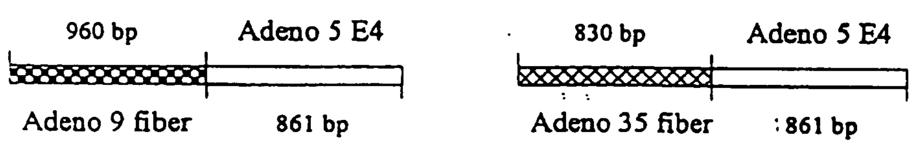

A. K562 cells
Number of viral genomes
I 2500
I 1250
I 625
I 315
Ad 9
I load 2100
I 0°C 1 h
I 37°C 2 h
MOI
I 10
I 105
I 420
I 2100
Ad 5
I load 2100
I 0°C 1 h
I 37°C 2 h
MOI
I 105
I 420
I 2100
Ad 35
I load 2100
I 0°C 1 h
I 37°C 2 h
MOI
I 10
I 105
I 420
I 2100
kb
35
12
6.1
5.8
1.8
kb
12
9.5

FIG. 15

B. CD 34+ cells



C.



Ad 5/9 DNA probe

Ad 5/35 DNA probe

FIG. 15 continued

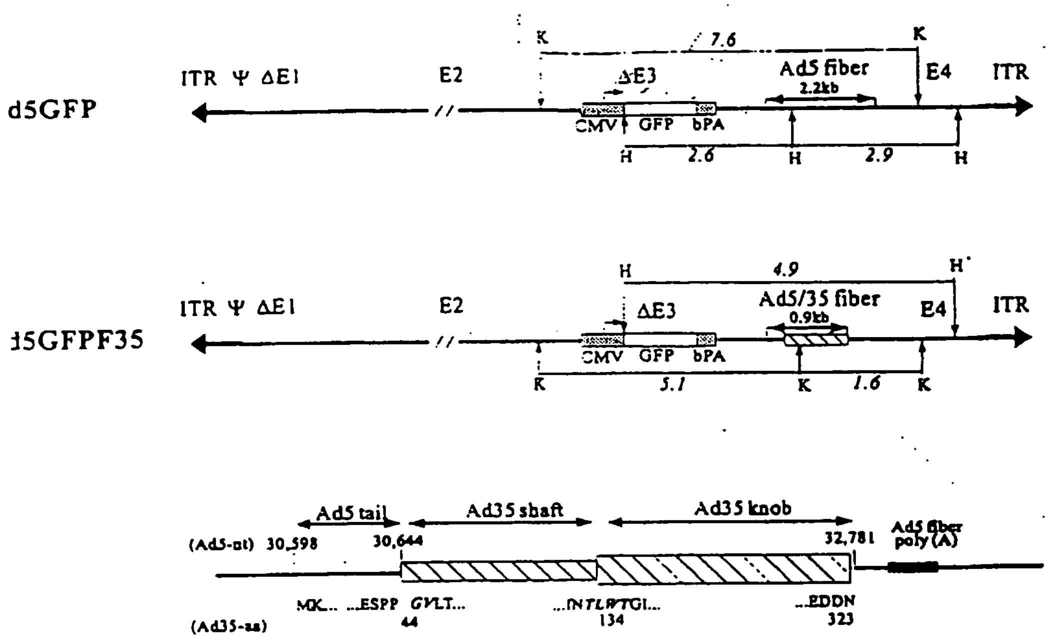


FIG 16A

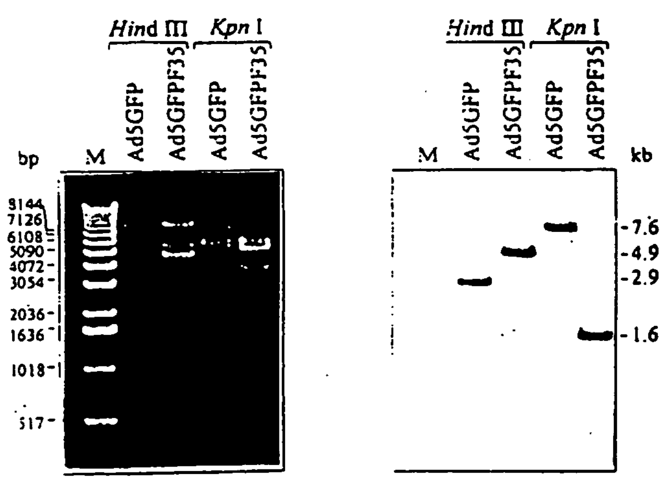


FIG. 16B

FIG. 16

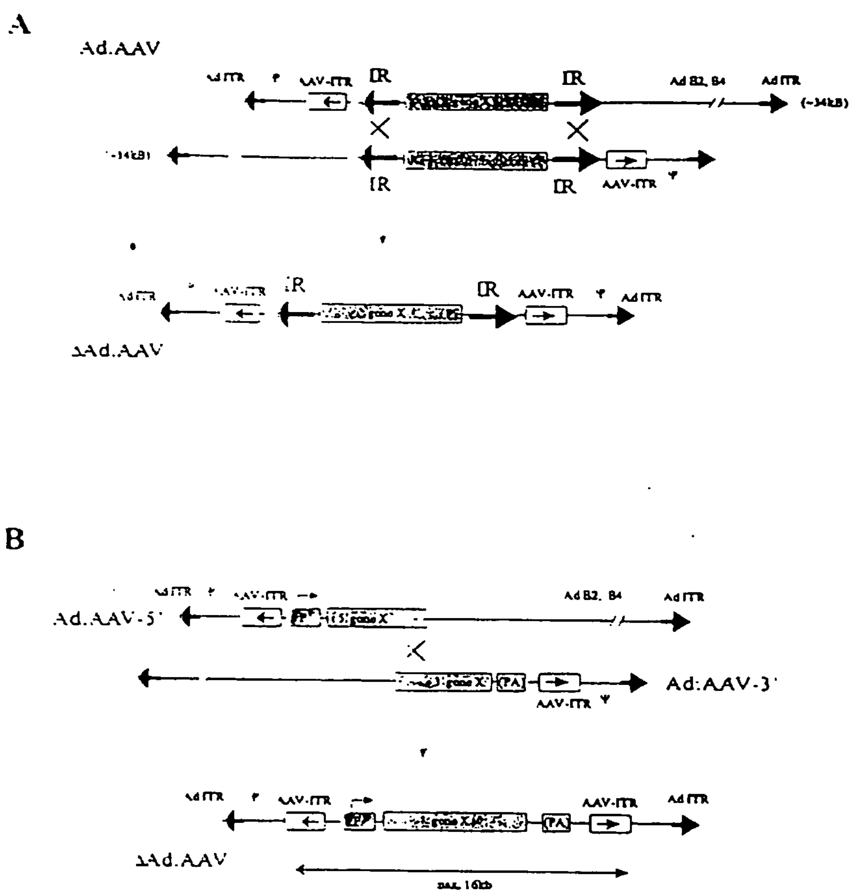

A
Ad.AAV
AAV-ITR
IR
Ad E2, E4
Ad ITR
IR
AAV-ITR
ΔAd.AAV
B
Ad.AAV-5'
gene X
Ad E2, E4
Ad ITR
gene X
PA
Ad.AAV-3'
AAV-ITR
ΔAd.AAV
max. 16kb

FIG. 17

Ad5.GFP/F35

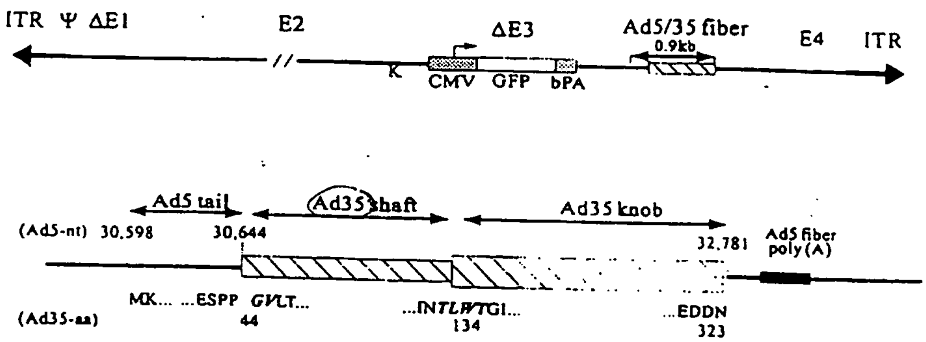


FIG. 18

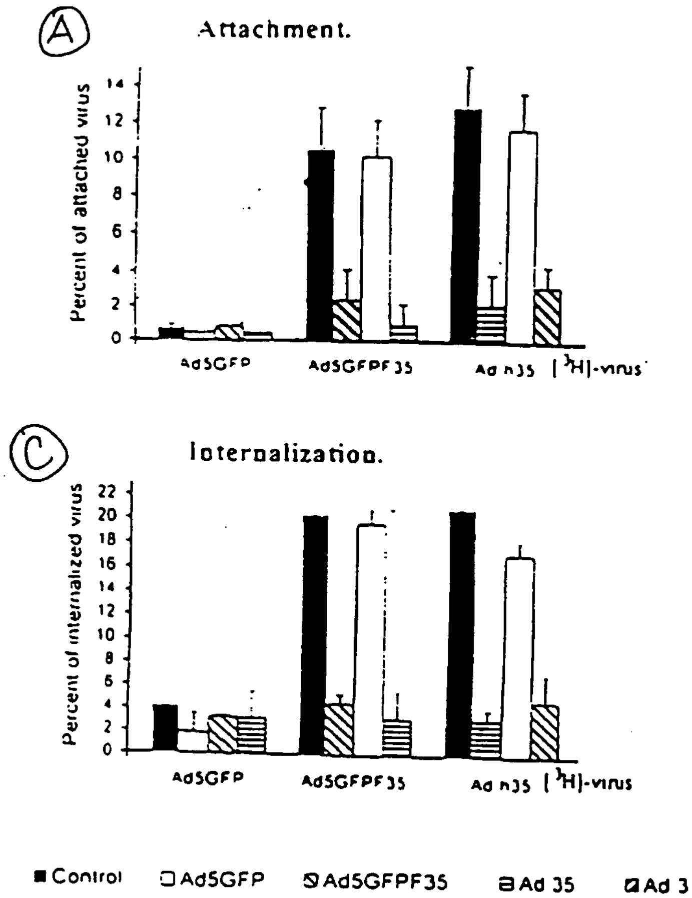

A
Attachment.
Percent of attached virus
14
12
10
8
6
4
2
0
Ad5GFP
Ad5GFPF35
Ad n35 [³H]-virus
B
Attachment.
Labeled adenovirus attachment (percent of control)
120
100
80
60
40
20
0
anti-CAR MAb
C
Internalization.
Percent of internalized virus
22
20
18
16
14
12
10
8
6
4
2
0
Ad5GFP
Ad5GFPF35
Ad n35 [³H]-virus
D
Internalization.
Labeled adenovirus internalization (percent of control)
120
100
80
60
40
20
0
L230 MAb
Control
Ad5GFP
Ad5GFPF35
Ad 35
Ad 3

FIG. 19

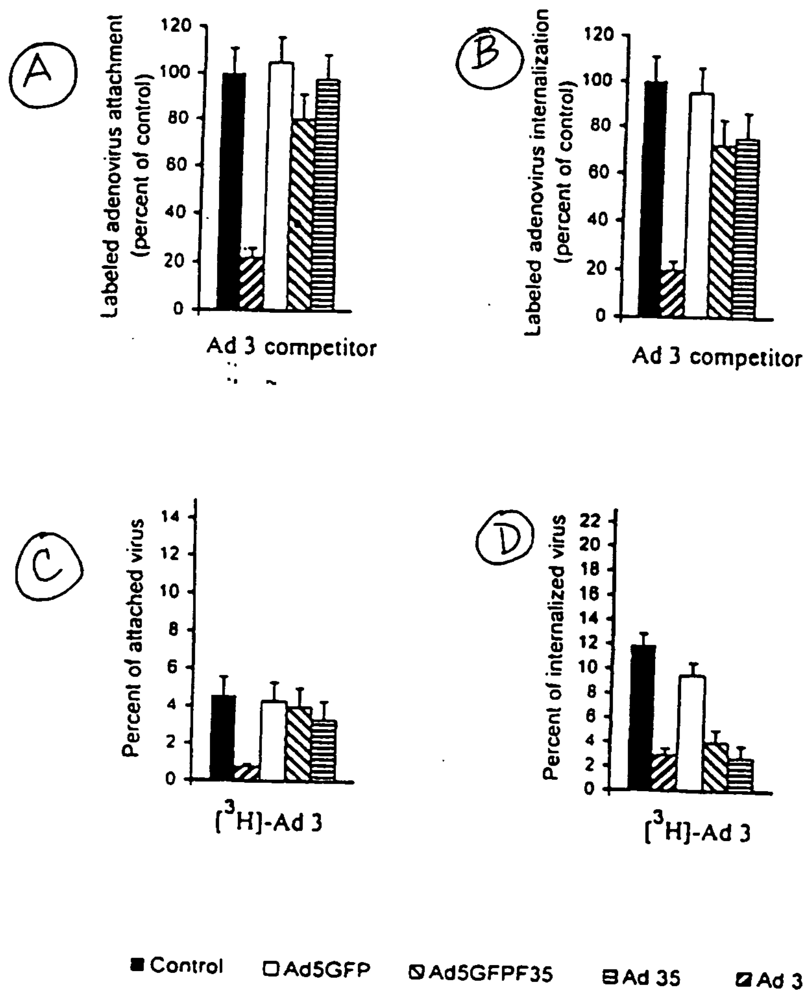
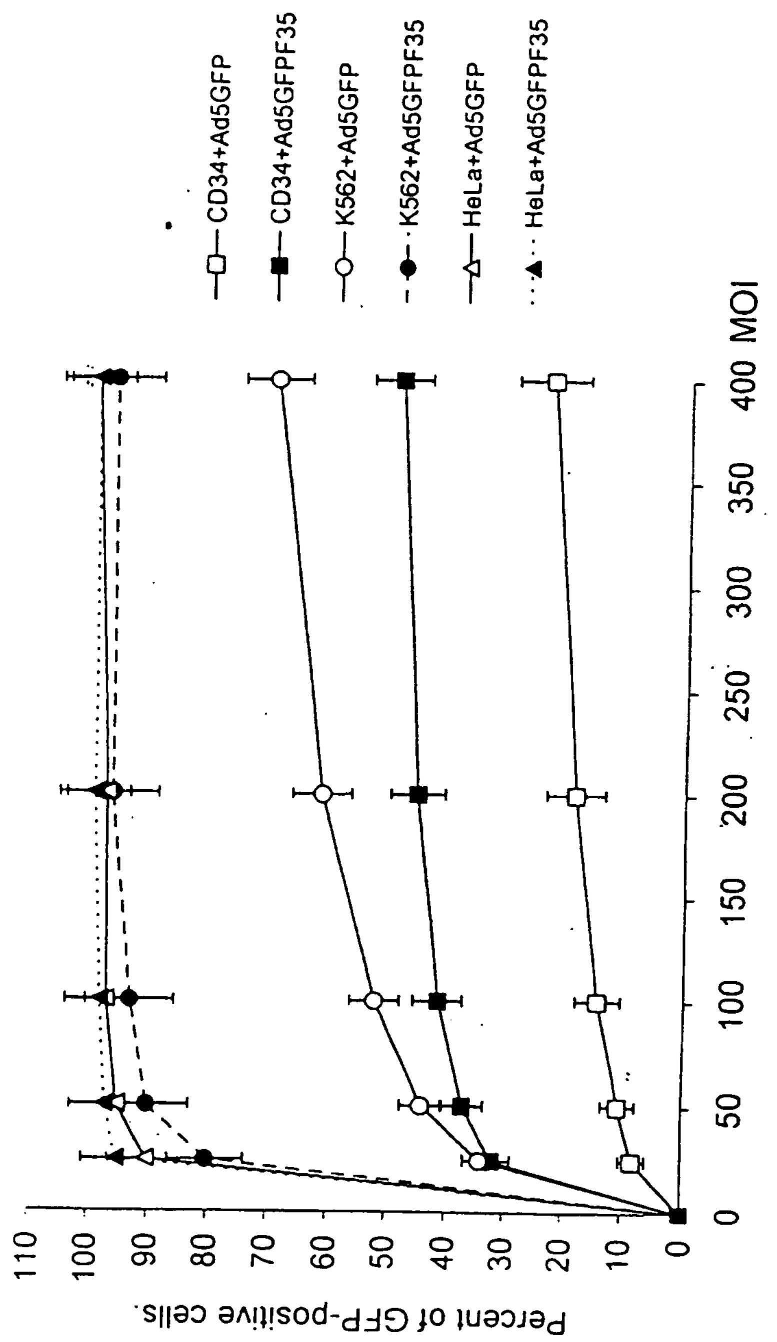
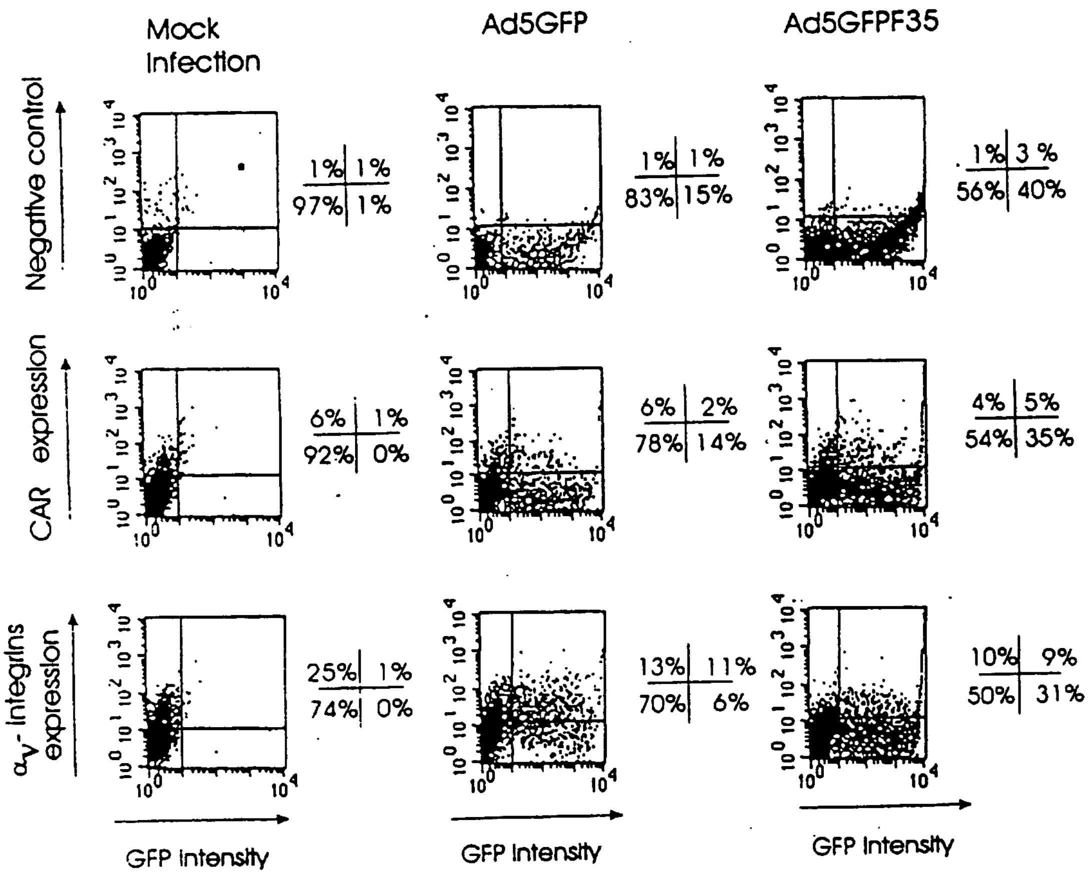

A
Labeled adenovirus attachment (percent of control)
120
100
80
60
40
20
0
Ad 3 competitor
B
Labeled adenovirus internalization (percent of control)
120
100
80
60
40
20
0
Ad 3 competitor
C
Percent of attached virus
14
12
10
8
6
4
2
0
[3H]-Ad 3
D
Percent of internalized virus
22
20
18
16
14
12
10
8
6
4
2
0
[3H]-Ad 3
Control
Ad5GFP
Ad5GFPF35
Ad 35
Ad 3

FIG. 20



CD34+Ad5GFP
CD34+Ad5GFPF35
K562+Ad5GFP
K562+Ad5GFPF35
HeLa+Ad5GFP
HeLa+Ad5GFPF35
Percent of GFP-positive cells.
MOI
0
10
20
30
40
50
60
70
80
90
100
110
0
50
100
150
200
250
300
350
400

FIG. 21



Mock Infection
Ad5GFP
Ad5GFPF35
Negative control
CAR expression
α_V- Integrins expression
1% 1%
97% 1%
1% 1%
83% 15%
1% 3 %
56% 40%
6% 1%
92% 0%
6% 2%
78% 14%
4% 5%
54% 35%
25% 1%
74% 0%
13% 11%
70% 6%
10% 9%
50% 31%
GFP Intensity
GFP Intensity
GFP Intensity

FIG. 22

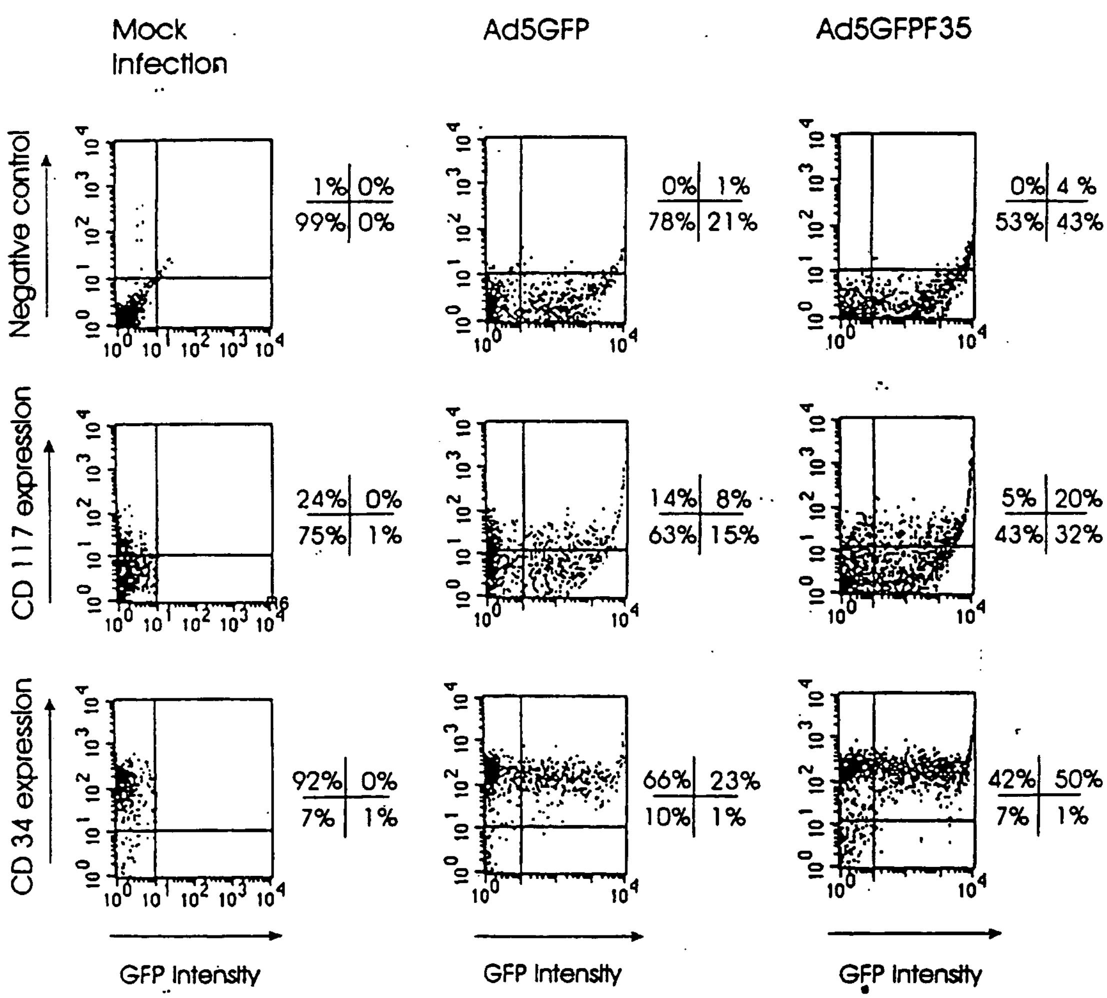

Mock Infection
Ad5GFP
Ad5GFPF35
Negative control
CD 117 expression
CD 34 expression
1% 0%
99% 0%
0% 1%
78% 21%
0% 4 %
53% 43%
24% 0%
75% 1%
14% 8%
63% 15%
5% 20%
43% 32%
92% 0%
7% 1%
66% 23%
10% 1%
42% 50%
7% 1%
GFP Intensity
GFP Intensity
GFP Intensity

FIG. 23 A

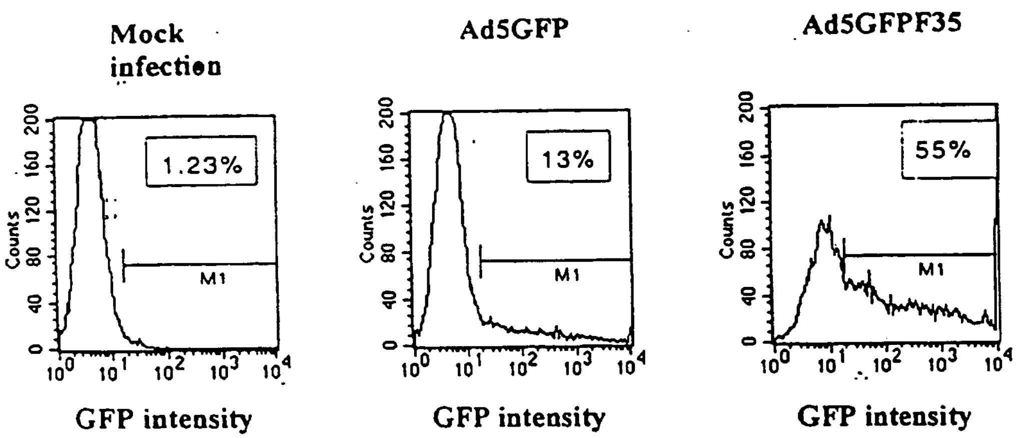

Mock infection
Ad5GFP
Ad5GFPF35
1.23%
13%
55%
M1
Counts
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4
GFP intensity

FIG 23 B

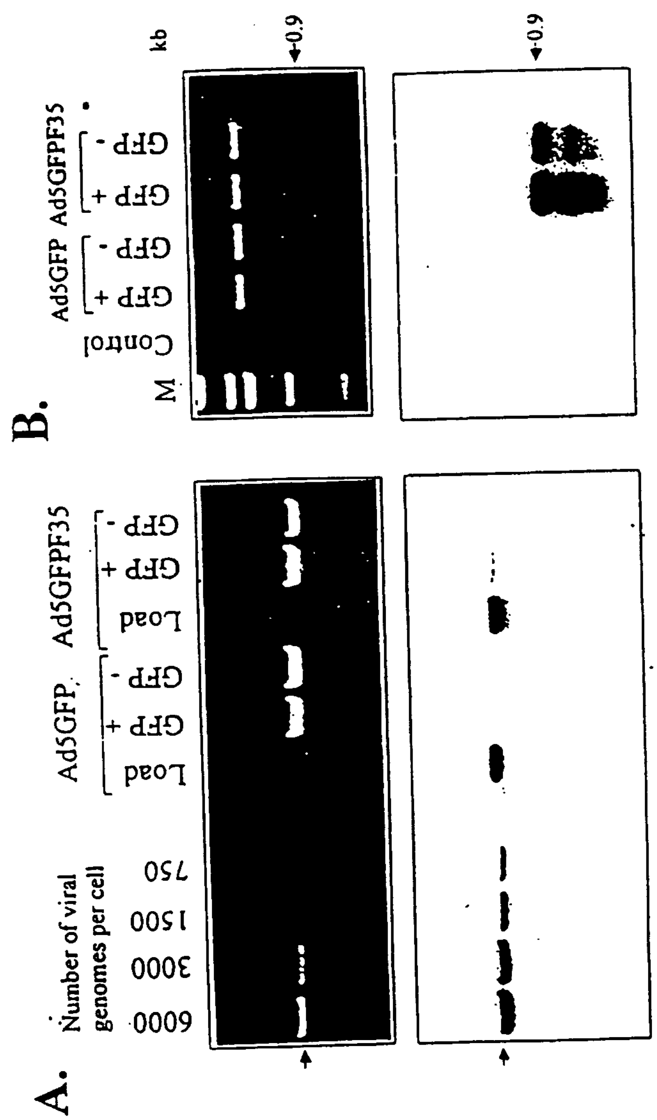
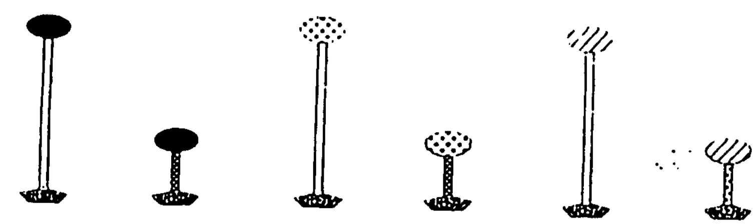

A.
Number of viral genomes per cell
6000
3000
1500
750
Ad5GFP
Load
GFP +
GFP -
Ad5GFPF35
Load
GFP +
GFP -
B.
M
Control
Ad5GFP
GFP +
GFP -
Ad5GFPF35
GFP +
GFP -
kb
0.9
0.9

FIG. 24

Structure and features of chimeric adenovirus vectors



Adeno vector	Ad 5L	Ad 5S	Ad 5/9L	Ad 5/9S	Ad 5/35L	Ad 5/35S
Binding to CAR	Yes	Yes	Yes	Yes	No	No
Genome/PFU ratio	13	143	29	582	17	14
Attachment to/ internalization into						
293	+++	++	++	+/- -	+++	+++
K562	- /+	- -/+	- -/+	- - -	+++	+++
Y79	++	+	+	+/-	+	+
Gene transfer efficiency						
293	+++	++	++	+/- -	+++	+++
K562	- /+	- -/+	- -/+	- - -	+++	+++
Y79	++	+	+	+/-	+	+

FIG. 25

Top view of Adenovirus type 5 fiber knob domain

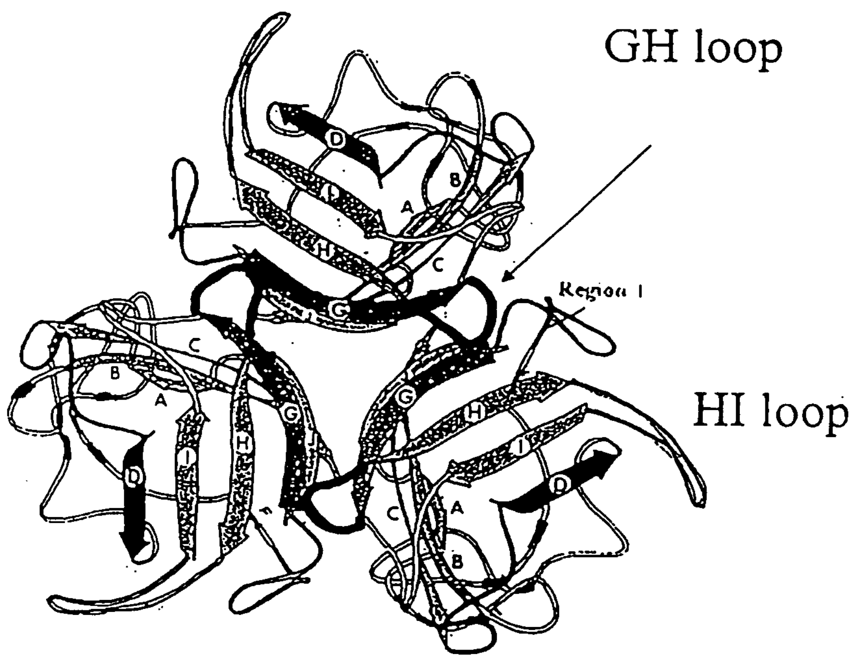
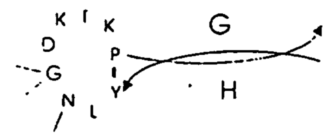


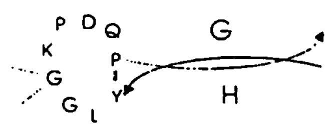
FIG. 26

Amino acids composition of wt and modified Adeno 5 GH loop

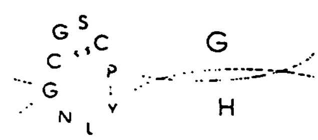
Adeno 5 wt GH-loop V Y] L N G D K T K [P V



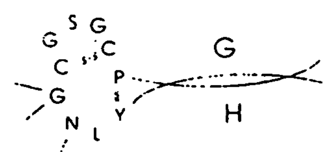
Adeno 5/9 GH-loop chimera V Y] L G G K P D Q [P V



Adeno 5 GH-Cys1 V Y] L N G C G S C [P V



Adeno 5 GH-Cys2 V Y] L N G C G S G C [P V



Adeno 5 GH-peptide V Y] L N G C G (X)n G C [P V

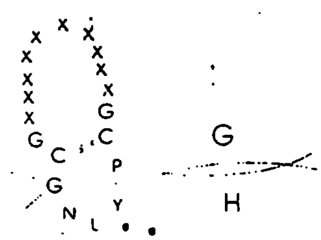


FIG. 27

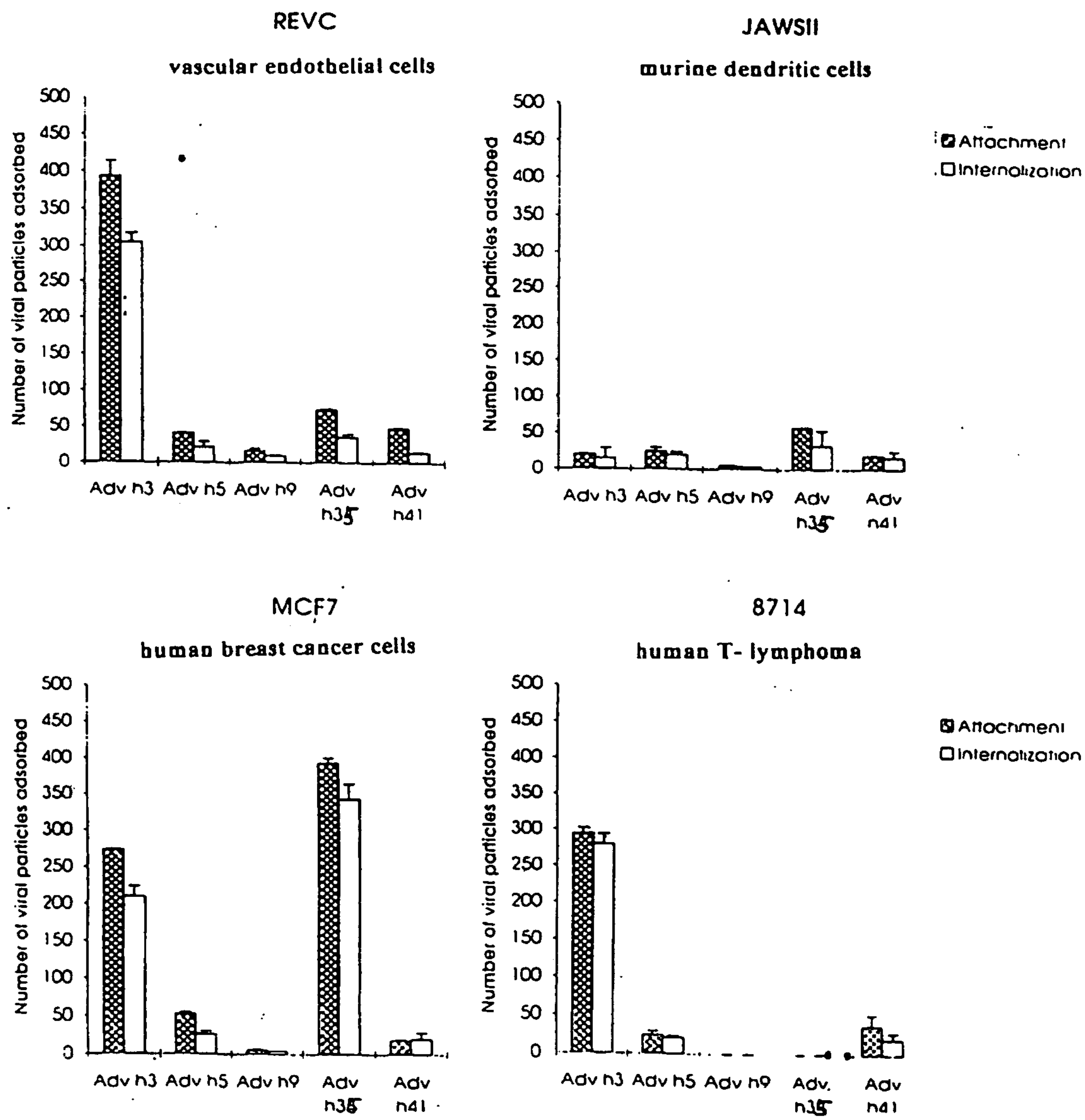

REVC
vascular endothelial cells
JAWSII
murine dendritic cells
MCF7
human breast cancer cells
8714
human T- lymphoma
Number of viral particles adsorbed
500
450
400
350
300
250
200
150
100
50
0
Adv h3
Adv h5
Adv h9
Adv h35
Adv h41
Attachment
Internalization

FIG 28

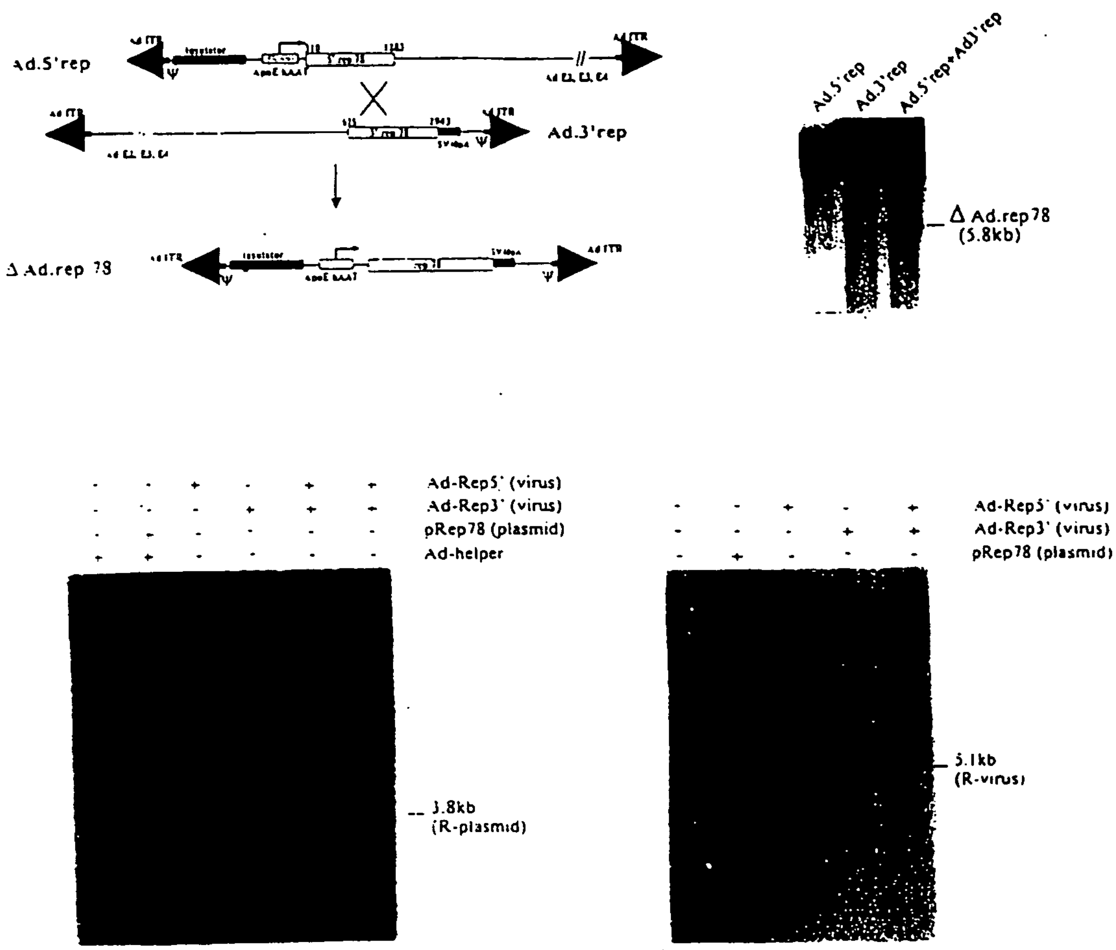

Ad.5'rep
Ad.3'rep
Δ Ad.rep 78
Ad.5'rep
Ad.3'rep
Ad.5'rep+Ad3'rep
Δ Ad.rep78
(5.8kb)
Ad-Rep5' (virus)
Ad-Rep3' (virus)
pRep78 (plasmid)
Ad-helper
3.8kb
(R-plasmid)
Ad-Rep5' (virus)
Ad-Rep3' (virus)
pRep78 (plasmid)
5.1kb
(R-virus)

FIG. 29

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/73478 A3

(51) International Patent Classification[7]: C12N 15/861, 15/864, 15/10, A61K 48/00

(21) International Application Number: PCT/US00/15442

(22) International Filing Date: 1 June 2000 (01.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/137,213 1 June 1999 (01.06.1999) US
60/161,097 22 October 1999 (22.10.1999) US

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF WASHINGTON** [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105-4631 (US).

(72) Inventors; and

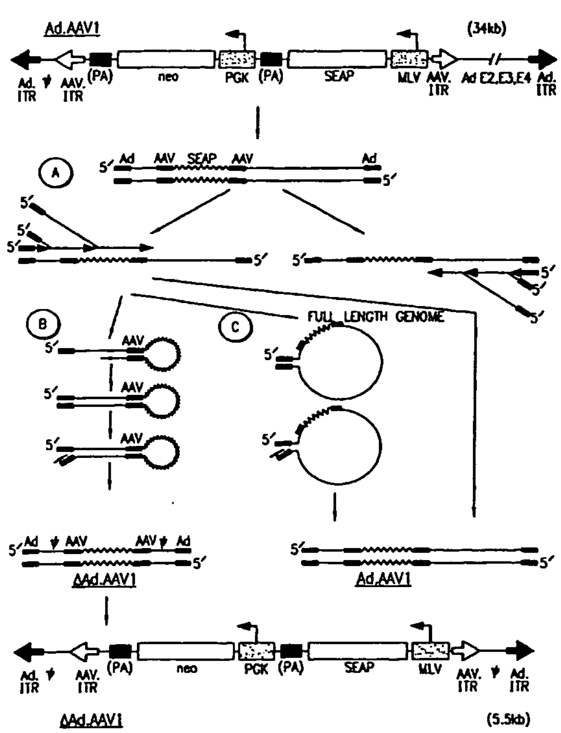
(75) Inventors/Applicants *(for US only)*: **LIEBER, André** [DE/US]; 7251 29th Avenue, Seattle, WA 98115 (US). **SHAYAKHMETOV, Dmitry** [RU/US]; 6500 25th Avenue, Seattle, WA 98115 (US). **FARRAR, Denise** [US/US]; 1324 W. Emerson Street, Seattle, WA 98119 (US). **PAPAYANNOPOULOU, Thalia** [US/US]; 702 35th Avenue, Seattle, WA 98122 (US).

(74) Agent: **ADRIANO, Sarah, B.**; Mandel & Adriano, Suite 60, 35 No. Arroyo Parkway, Pasadena, CA 91103 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID,

[Continued on next page]

(54) Title: RECOMBINANT ADENOVIRAL VECTORS EXPRESSING CHIMERIC FIBER PROTEINS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION



(57) Abstract: The present invention provides for chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_v\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

WO 00/73478 A3

IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) **Date of publication of the international search report:**
5 July 2001

(48) **Date of publication of this corrected version:**
24 January 2002

(15) **Information about Correction:**
see PCT Gazette No. 04/2002 of 24 January 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

RECOMBINANT ADENOVIRAL VECTORS EXPRESSING CHIMERIC FIBER PROTEINS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION

FIBER PROTEINS

This invention was made, at least in part, with funding from the National Institutes of Health (Grant Nos. R01 CA 80192-01 and R21 DK 55590-01). Accordingly, the United States Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the field of gene therapy, and in particular, to novel adenovirus (Ad) vectors that selectively infect cells for gene therapy, and to Ad vectors containing modifications of the fiber protein to allow retargeting of any adenovirus serotype.

BACKGROUND OF THE INVENTION

Gene transfer vectors require the efficient transduction of target cells, stable association with the host genome, and adequate transgene expression in the appropriate target cell, without associated toxic or immunological side effects,. Currently available viral vector systems, including recombinant retroviruses, adenoviruses and adeno-associated viruses, are not suitable for efficient gene transfer into many cell types. Retroviral vectors require cell division for stable integration. Recombinant adenoviruses are not able to infect many cell types important for gene therapy, including hematopoietic stem cells, monocytes, T- and B-lymphocytes. Moreover, recombinant adeno-associated vectors (AAV) integrate with a low frequency.

First generation adenoviruses have a number of properties that make them an attractive vehicle for gene transfer (Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205). These include the ability to produce purified virus at high titers in concert with highly efficient gene transfer of up to 8 kb long expression cassettes into a large variety of cell

types *in vivo*, including non-dividing cells. Limitations of first generation adenoviruses include the development of immune responses to expressed viral proteins resulting in toxicity and virus clearance. The episomal status of adenoviral DNA within transduced cells is another limitation of first generation Ad vectors. Stable integration of adenovirus DNA into the host genome is reported only for wild-type forms of specific subtypes and appears not to occur in a detectable manner with E1/E3-deleted Ad 5 (adenovirus serotype 5) vectors widely used for gene transfer *in vitro* and *in vivo* [Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205].

Recombinant AAV vectors (rAAV) integrate with a low frequency (about 1 out of 20,000 genomes) randomly as cocatemers into the host genome (Rutledge, E.A.; Russel, D.W. 1997 *J. Virology*, 71, 8429-8436). The presence of two AAV inverted terminal repeats (ITRs) and as yet unknown host cellular factors seem to be the only requirement for vector integration (Xiao, X., et al, 1997, *J. Virology*, 71, 941-948; Balague, C., et al. 1997, *J. Virology*, 71, 3299-3306; Yang, C.C. 1997, *J. Virology*, 71, 9231-9247). In the presence of the large AAV Rep proteins, AAV integrates preferentially into a specific site at human chromosome 19, called AAVS1 (Berns, K.I., 1996, *Fields Virology*, Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2173-2220). The AAV capsid is formed by three coat proteins (VP1-3), which interact with specific heparin sulfates on the cell surface and probably with specific receptor(s). However, many cell types, including hematopoietic stem cells, lack these structures so that rAAV vectors based on AAV2 cannot infect or transduce these cells (Malik P. et al., 1997, *J. Virology*, 71, 1776-1783; Quing, K.Y., et al. 1998, *J. Virology*, 72, 1593-1599). Other disadvantages of rAAV vectors include the limited insert size (4.5-5kb) that can be accommodated in rAAV vectors lacking all viral genes and low transducing titers of rAAV preparations.

Adenovirus infection is initiated by attaching to the cell surface of Ad 5 via its fiber protein (for a review, see Shenk, T. 1996 *Fields Virology, Vol. 2*, Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2111-2148). The distal, C-terminal domain of the trimeric fiber molecule terminates in a knob, which binds to a specific cellular

receptor identified recently as the coxackie-adenovirus receptor (CAR) (Bergelson, J.M. et al. *Science*, 275, 1320-1323). After binding, in an event independent of virus attachment, Arg-Gly-Asp (RGD) motifs in the penton base interact with cellular integrins of the $\alpha 3$ and $\beta 5$ types. This interaction triggers cellular internalization whereby the virion achieves localization within the endosome. The endosomal membrane is lysed in a process mediated by the penton base, releasing the contents of the endosome to the cytoplasm. During these processes, the virion is gradually uncoated and the adenoviral DNA is transported to the nucleus where replication takes place. The terminal protein, which is covalently attached to the viral genome and the core protein V that is localized on the surface of the cores have nuclear localization signals (NLSs) (van der Vliet, B. 1995, *The Molecular Repertoir of Adenoviruses, Vol. 2*, Doerfler, W. and Boehm, P.(ed.), Springer Verlag, Berlin, 1-31). These NLSs play a crucial role in directing the adenoviral genome to the nucleus and probably represent the structural elements which allow adenovirus to transduce non-dividing cells. When the double-stranded, linear DNA reaches the nucleus, it binds to the nuclear matrix through its terminal protein.

Since the cell types that can be infected with Ad5 or Ad2 vectors are restricted by the presence of CAR and specific integrins, attempts have been made to widen the tropism of Ad vectors. Genetic modification of adenovirus coat proteins to target novel cell surface receptors have been reported for the fiber (Krasnykh, V. et al. 1998 *J. Virology*, 72, 1844-1852, Krasnykh, V. et al. 1996 *J. Virology*, 70, 6839-6846, Stevenson, S.D., et al. 1997, *J. Virology*, 71, 4782-4790), penton base (Wickham, T.J., et al. 1996, *J. Virology*, 70, 6831-6838; Wickham, T.J., et al. 1995, *Gene Therapy*, 69, 750-756), and hexon proteins (Crompton, J., et al. 1994, *J. Gen. Virol.* 75, 133-139). The most promising modification seems to be the functional modification of the fiber protein or more specifically of the fiber knob as the moiety, which mediates the primary attachment. Two groups have reported the generation of fibers consisting of the Ad5 tail/shaft and the knob domain of Ad3 (Krasnykh, V. et al. 1996 *supra*, Stevenson, S.D., et al. 1997, *supra*). Recently, recombinant adenoviruses with fibers containing C-terminal poly-lysine, gastrin-releasing peptide, somatostatin, E-selectin-binding peptide, or oligo-Histidines were produced in order to change the native tropism of Ad5. Krasnikh et al. found (Krasnykh, V. et al.

1998 *supra*) that heterologous peptide ligands could be inserted into the H1 loop of the fiber knob domain without affecting the biological function of the fiber. Based on studies with other Ad serotypes, it appears that the length of the fiber shaft is a critical element, determining the efficiency of interaction with cell surface integrins and the internalization process. Thus far, there is no reported data demonstrating successful retargeting of Ad5 vectors for a specific cell type.

Therefore, there is a present need for an improved adenovirus vector which can be targeted efficiently to a variety of cell types and tissues and remain stably integrated in the host genome with minimal antigenicity to the host. The present invention discloses novel chimeric adenoviral (Ad) Ad-AAV vectors, which express a modified fiber protein on their capsid, for specifically targeting the vector. Methods of making, uses and advantages of these vectors are described. In addition, the alteration described for the knob and shaft domains of the fiber protein provide a novel approach to retarget any adenovirus serotype for cell specific infection.

SUMMARY OF THE INVENTION

The present invention provides for novel chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_{\upsilon}\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

The recombinant adenovirus vectors of the invention (Example I) provide a novel design that allows for the easy production and delivery of a "gutless" adenoviral vector with the added advantage of stable integration of the transgene into the host genome of different cell type. The adenoviral vector described is devoid of all adenoviral sequences except for the 5' and 3' cis elements necessary for replication and virion encapsidation. The adenovirus-associated virus sequences of the invention comprising the 5' (right) and 3' (left) inverted terminal repeats (ITRs) flank the transgene gene cassette such that they

direct homologous recombination during viral replication and viral integration into the host genome. In one embodiment AAV-ITR flanking sequences are used. The vector also contains a selected transgene(s) operably linked to a selected regulatory element and a polydenylation stop signal, which is in turn flanked by the flanking sequences described above. The selected transgene(s)can be linked under the same regulatory elements or under separate regulatory elements in the same orientation or in opposite orientations with respect to each other. The selected transgene(s) are any gene or genes which are expressed in a host cell or tissue for therapeutic, reporter or selection purposes. This vector is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host genome. Also provided is a method to improve the integration frequency and site specific integration by incorporating an AAV rep protein into the recombinant hybrid vector.

The invention also provides chimeric fiber proteins (Example II), which includes naturally occurring fiber proteins in which a portion or portions of the sequence are modified to alter cell or tissue specificity of infection. Altered fiber protein sequences can include fiber protein domains (the knob domain, the shaft domain, and the tail domain) from other or the same adenovirus serotypes or from randomly selected peptides. A chimeric fiber protein can be entirely composed of non-naturally occurring sequences. The invention further relates to nucleic acid sequences encoding the chimeric fiber proteins. These nucleic acid sequences can be naturally occurring, a mixture of naturally occurring and non-naturally occurring sequences, or entirely non-naturally occurring sequences.

The heterologous fiber protein sequences described herein can be inserted into any adenovirus based vector which contains a capsid, rendering the virus capable of specifically infecting a given cell or tissue. Adenoviral vectors having such a heterologous fiber sequence can be used to direct gene transfer into desired cells. For stable integration of the transgene cassette into the host gemone, the chimeric Ad-AAV vector described in the invention is the preferred vector of use.

The invention also includes a library of adenoviruses displaying random peptides in their fiber knobs can be used as ligands to screen for an adenovirus variant with tropism to a particular cell type *in vitro* and *in vivo*.

The chimeric Ad- vectors described herein include the Ad.AAV genome with a modified fiber protein expressed on its capsid. These chimeric vectors are designed to infect a wide variety of cells, in particular, the cells which can only be poorly transduced by the commonly used retroviral, AAV and adenoviral vectors. These cells include, but not limited to, hematopoietic stem cells, lung epithelial cells, dendritic cells, lymphoblastoid cells, and endothelial cells. Hematopoietic stem cells such as CD34+ cells can be targeted for gene therapy of sickle cell anemia and thalasemia using the vector described herein. The chimeric Ad-AAV vector capable of transducing genes into endothelial cells can be used in gene therapy for vascular diseases such as atherosclerosis or restinosis after coronary artery surgery.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1C display a proposed mechanism for forming of ΔAd.AAV1 genome.

Figs. 2A and 2B show electron photomicrographs of hybrid virus particles: Fig. 2A shows Ad.AAV1 and Fig. 2B shows ΔAd.AAV1.

Fig. 3 illustrates analysis of ΔAd.AAV1 genomes after transduction of SKHep1 cells. Pulse field gel eletrophoresis (PFGE). $1x10^6$ control Sk Hep1 cells (SKHep1) (lanes 1-3, 5, 9). SKHep1 cells from G418 resistant pools (ΔAd.AAV1) (infected with ΔAd.AAV1 and selected for 4 weeks) (lanes 6-8, 10-12), or SKHep1 cells collected at 3 days after infection with 2000 genomes Ad.AAV1 (Ad) lanes 4, 13) are sealed in agarose plaques, lysed *in situ* and subjected to PFGE with or without prior digestion with restriction endonucleases. Southern Blot is performed with a SEAP specific probe. U = undigested, P = digested with PI- Sce1, I = I-CeuI, E = EcoREI.

Figs. 4A and 4B show response of K562 and CD34+ cells respectively after infection with ΔAd.AAVBG. Cells are incubated for 6 hours with virus under agitation. At day 3 after infection, transduction frequency is calculated based on the number of X-Gal positive cells. Viability is tested by trypan blue exclusion. N = 3, SEM < 10%.

Fig. 5 shows Rep expression in SKHep1 and 293 cells after plasmid transfection. $5x10^5$ cells are transfected with pAAV/Ad, pRSVrep, or pPGKrep by Ca-phosphate co-precipitation. Three days after transfection, cells are harvested. Lysates are separated on a 10% PA gel, followed by Western Blot with Rep specific antibodies (03-65169), American Research Products), and developed with ECL (Amersham).

Fig. 6 shows detection of vector integration into AAVS1 by PFGE.

Fig. 7 shows strategy for creating an ΔAd.AAV hybrid vector capable of site-specific integration. Arrows indicate promoters, (PA) = polyadenylation signal. Ψ = adenoviral packaging signal.

Fig. 8A-8B shows vectors for transduction studies with SNori as expression unit and analysis of vector integration on genomic DNA from a small cell number. Analgous vector sets can be generated with β-galactosidase (BG) or green fluorescence protein (GFP) as reporter genes.

Fig. 9 shows strategy for substituting the Ad5 fiber sequence by the heterologous fiber X genes using recombination in *E. coli.*

Fig. 10 shows the expression of CAR and α_v-integrins on test cells. For flow cytometry analysis, HeLa, CHO, K562, and CD34+ cells were incubated with monoclonal anti-CAR (RmcB, 1:400 dilution) or anti-α_v-integrin antibodies (L230, 1:30 dilution). As a negative control, cells were incubated with an irrelevant mouse monoclonal antibody (anti-BrdU, 1:100 dilution). The binding of primary antibodies was developed with anti-mouse IgG-

FITC labeled conjugates (1:100 dilution). Data shown represent the average results of quadruplicate analyses performed on 10^4 cells.

Fig. 11 shows the electron microscopy of adenovirus particles. Purified particles from Ad5, 9, and 35 were negative contrast stained and analyzed at a magnification of 85,000x. Defective particles are highlighted by arrows.

Fig. 12 shows the analysis of attachment and internalization of different serotypes to CHO, HeLa, K562, and CD34+ cells. Equal amounts of [^{3}H]-thymidine-labeled virions of Ads 3, 4, 5, 9, 35, and 41 (measured by OD_{260}, and equivalent to an MOI of 400 pfu per cell for Ad5) were incubated for one hour on ice as described in Materials and Methods. Cells were then washed, and the number of labeled virions bound per cell was determined. For internalization studies, viruses were first allowed to attach to cells for 1 h on ice. Then, unbound viral particles were washed out. Cells were then incubated at 37°C for 30 min followed by treatment with trypsin-EDTA and washing to remove uninternalized viral particles. The data were obtained from two to four independent experiments performed in triplicate. Note the different scale on the Y-axes for CD34+ cells.

Figs. 13A-13C show attachment and internalization of different adenovirus serotypes to Hela, CHO and 293 cells respectively.

Figs. 14A and 14B show attachment and internalization of different adenovirus serotypes to CD34+ and K-562 cells respectively.

Fig. 15A-15C shows the analysis of viral replication in K562 and CD34+ cells by Southern blot analysis of methylated viral DNA. Replication studies were performed with $1x10^5$ K562 cells (A) or CD34+ cells (B), infected with methylated Ad5, Ad9 or Ad35. The lane labeled as "load" represents DNA that was extracted form the media/cell mixture immediately after adding the indicated viral dose to cells. The intensities of bands corresponding to methylated and un-methylated viral DNA indicate that ~85% of

the input virus was methylated. To quantify adsorption and internalization, DNA analysis was performed after prior incubation of virus with cells at 0^0C (adsorption) or 37^0C (internalization). For dose dependent replication studies, the indicated viral dose (expressed as the number of genomes) was added to the cells, and cellular genomic DNA together with viral DNA was extracted 16 hours or 36 hours post-infection for K562 and CD34+cells, respectively. Identical amounts of sample DNA were analyzed by Southern blot. For quantification purposes, Ad9 replication was analyzed together with Ad5 using an Ad5/9 chimeric probe that hybridizes with DNA of both serotypes (C). The analysis of Ad5 versus Ad35 replication was performed with the corresponding Ad5/35 chimeric probe. Since separate hybridizations with both Ad5/35 and Ad5/9 probes gave identical signal intensities for Ad5 DNA only one panel is shown for Ad5 replication in test cells. To produce distinguishable fragments specific for the methylated or non-methylated status of viral genomes, Ad5 DNA was digested with *Xho* I, while Ad9 and Ad35 DNA was digested with *Xho* I and *Hin*d III. The bands specific for methylated (not-replicated) viral DNA were ~12kb for Ad9, 35kb for Ad5, and ~12kb for Ad35. The fragments specific for non-methylated DNA were 5.8kb for Ad9, 6.1kb for Ad5, and 9.5kb for Ad35. Chimeric Ad5/9 and Ad5/35 DNA fragments (1.8kb) were used as quantification standards and applied onto gel together with digested viral/cellular DNA (shown on the left part of the figures).

Fig. 16A-16B shows the structure of Ad5GFP and chimeric Ad5GFP/F35 vectors. A) Schematic diagram of the original E1/E3 deleted Ad5-based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.coli. *Kpn* I (K) and *Hin*d III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes. A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The

Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal. The region of Ad5GFP/F35 encoding for chimeric fiber was completely sequenced with Ad5 specific primers (see Material and Methods). B) Restriction analysis of viral genomes. Viral DNA was isolated from purified Ad5GFP and Ad5GFP/F35 particles as described elsewhere. One microgram of DNA was digested with *Hin*d III or *Kpn* I and separated in ethidium bromide stained agarose gels (left panel) which were subsequently blotted and analyzed by Southern blot with an Ad5 E4 specific probe (nt 32,7775-33,651) (right panel). Specific patterns, designating the correct structure for both viral vectors were detected. The *Hin*d III fragments specific for Ad5GFP and Ad5GFP/F35 were 2.9kb and 4.9kb, respectively. The *Kpn* I fragment that confirmed the correct Ad5GFP/F35 structure was 1.6kb compared to a 7.6kb Ad5GFP fragment. M - 1kb ladder (Gibco-BRL, Grand Island, NY).

Fig. 17 shows the generation of ΔAd.AAV genenomes by recombination between inverted homology regions. Recombination between two inverted repeats (IR) present in one Ad.AAV vector. The first-generation Ad.AAV vector (~34kb) contains two 1.2kb inverted homology elements flanking the transgene cassette. One AAV-ITR is inserted between the Ad packaging signal (ψ) and the left IR. During Ad replication, recombination between the Irs mediates the formation of the ΔAd.AAV genomes with the transgene flanked by Irs, AAVITRs, Ad packaging signals, and Ad ITRs. These genomes are efficiently packaged into Ad capsids.

Fig. 18 shows the structure of Ad5/11, Ad5/35. Schematic diagram of the original E1/E3 deleted Ad50based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.col. Kpn I (K) and Hind III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes.

A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal.

Fig. 19 shows the cross-competition for attachment and internalization of labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled viruses, and with anti-CAR or anti-α_v-integrins Mab. (A) For attachment studies, 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled competitor virus at 4°C for 1 h. Then, equal amounts of [^{3}H]-labeled viruses, at a dose equivalent to an MOI of 100 pfu per cell determined for Ad5GFP, were added to cells followed by incubation at 4°C for 1 h. Cells were then washed with ice-cold PBS, pelleted and the percentage of attached virus (cell-associated counts per minute) was determined. For analysis of cross-competition for internalization, cells were pre-incubated with a 100-fold excess of competitor virus at 37°C for 30 min before labeled virus was added. After an additional incubation at 37°C for 30 min, cells were treated with trypsin-EDTA for 5 min at 37°C, washed with ice-cold PBS, pelleted, and the percentage of internalized virus was determined. For controls, cells were incubated with labeled viruses without any competitors. Preliminary experiments had shown that the conditions chosen for competition studies allowed for saturation in attachment/internalization on K562 cells for all unlabeled competitors. (B) 10^5 K562 cells were pre-incubated for 1 hour at 4°C with anti-CAR MAb (RmcB, diluted 1:100) or with anti-α_v-integrin MAb (L230, diluted 1:30), followed by incubation with labeled viruses according to the protocols for attachment or for internalization as described above. For each particular serotype, the percentage of attached/internalized virus was compared to the control settings, where cells were preincubated under the same conditions with a 1:100 dilution of an irrelevant antibody (anti-BrdU Mab) before addition of the labeled virus. Note that the specific competitors but not the corresponding controls significantly inhibited Ad5 internalization to a degree that is in agreement with published data (59). N>/=4

Fig. 20. Cross-competition for attachment and internalization of [^{3}H]-labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled Ad3 virus (A), and of [^{3}H]-

labeled Ad3 virions with unlabeled viruses (B). 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled viral particles according to attachment or internalization protocols described for Fig.6. Equal amounts of [^{3}H]-labeled Ad5GFP, Ad5GFP/F35, or Ad35 (A) or [^{3}H]-labeled Ad3 (B) were added to cells at a dose equivalent to an MOI of 100 pfu per cell for Ad5GFP. In control settings, cells were incubated with labeled viruses without any competitors. N=4.

Fig. 21 shows the transduction of CD34+, K562, and HeLa cells with Ad5GFP and chimeric Ad5GFP/F35 vectors. $1x10^5$ cells were infected with different MOIs (pfu/cell) of viruses in 100 μl of media for 6 hours at 37°C. Virus containing media was then removed, and the cells were resuspended in fresh media followed by incubation for 18 h at 37°C. The percentage of GFP expressing cells was determined by flow cytometry. N=3

Fig. 22 shows the distribution of GFP-positive cells in subpopulations of human CD34+ cells expressing CAR or α_v-integrins. $1x10^5$ CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu/cell as described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CAR (1:100 final dilution) or anti-α_v-integrin (1:30 final dilution) primary MAbs for 1 h at 37°C. Binding of primary antibodies was developed with anti-mouse IgG-PE labeled secondary MAbs (1:100 final dilution) at 4°C for 30 min. For each variant, 10^4 cells were analyzed by flow-cytometry. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The data shown were representative for three independent experiments.

Fig. 23A-23B shows the distribution of GFP-positive cells in a subpopulation of human CD34+ cells, expressing CD34 and CD117 (c-kit). (A) Co-localization of GFP expression with CD34 or CD117: CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell under the conditions described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CD34 PE-conjugated MAbs (final dilution 1:2) or with anti-CD117 PE-conjugated MAbs (final dilution 1:5)

for 30 min on ice, and 10^4 cells per variant were subjected to two-color flow cytometry analysis. For negative control staining, no antibodies were added to the cells before analysis. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The experiment was performed two times in triplicates, and typically obtained results are shown. The SEM was less than 10% of the statistical average. (B) Transduction of CD34+/CD117+ cells with Ad5GFP and chimeric Ad5GFP/F35 virus vectors: CD34+ cells, cultured overnight before staining in media without SCF, were incubated with PE-labeled anti-CD117 MAb for 30 min on ice. The fraction of CD117-positive cells was sorted by FACS. More than 97% of sorted cells were positive for CD117. $1x10^5$ CD117+/CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell, as for Fig.8. Twenty-four hours post infection, the percentage of GFP positive was determined by flow cytometry. For mock infection, CD117+/CD34+ cells were incubated with virus dilution buffer only. The infections were done in triplicates, and the average percentage of GFP-expressing cells is indicated on the corresponding histogram. The SEM was less than 10% of the statistical average.

Fig. 24 shows the southern analysis of viral genomes in GFP-positive and GFP-negative fractions of CD34+ cells infected with the Ad5GFP and chimeric Ad5GFP/F35 vectors. CD34+ cells were infected with viruses at an MOI of 100 as described for Fig.21. Twenty four-hours post infection, cells were sorted by FACS for GFP positive and GFP negative fractions. 10^5 cells from each fraction were used to isolate genomic DNA together with viral DNA. Before cell lysis, a rigorous treatment with trypsin and DNase followed by washing was performed to exclude that genomic DNA samples were contaminated by extracellular viral DNA. A) The upper panel shows the ethidium bromide stained 1% agarose gel before blotting demonstrating that similar amounts of genomic DNA were loaded. This amount corresponded to DNA isolated from ~25,000 GFP+ or GFP- cells. The lane labeled Aload@ represents viral DNA purified from Ad5GFP or Ad5GFP/F35 virions mixed with pBluescript plasmid DNA (Stratagene) as a carrier and applied on a

gel at the amount that was actually used to infect 25,000 cells. As a concentration standard, a serial dilution of Ad5GFP genomes was loaded on the gel (left side). For Southern analysis (lower panel), an 8kb-long HindIII fragment corresponding to the E2 region of Ad5 was used as a labeled probe. Hybridized filters were subjected to PhosphoImager analyis and then exposed to Kodak-X-OMAT film for 48 h at B70°C. The cellular/viral genomic DNA is indicated by an arrow. (B) To detect Ad5GFP genomes in transduced cells, PCR amplification followed by Southern blot hybridization was performed on the same samples that were used for quantitative Southern blot hybridization in (A). DNA purified from ~2,500 cells was subjected to PCR (95°CB1min, 53°C-1min, 72°CB 1min, 20 cycles with primers Ad5-F1 and Ad5-R1). One fifth of the PCR reaction was subjected to agarose gel electrophoresis (upper panel). A 0.9 kb-long DNA fragment, specific to the E4 region of Ad5 was detected for transduced Ad5GFP/F35 genomes. DNA then was blotted onto Nybond-N+ membrane and Southern blot hybridization (lower panel) with an Ad5 E4 specific DNA probe was performed. In addition to the 0.9kb DNA fragment, the PCR primers generated a smaller 0.5 kb-long fragment that also hybridized with with the E4 region probe.

Fig. 25 shows the role of fiber shaft length in Ad infection strategies. CAR binding (Ad5 and Ad9) variants and Ad35, which interacts with a non-CAR receptor were analyzed on CAR expressing cells (293, Y79) and K562 cells which do not express significant CAR amounts. All vectors contain a GFP expression cassette packaged into an Ad5 capsid with modified fibers.

Fig. 26 shows the tertiary structure of Ad5 knob: localization of CAR binding sites, H-I loop and G-H loop.

Fig. 27 shows the substitution of the G-H loop with heterologous peptides.

Fig. 28 shows the attachment and internalization of metabolically labeled serotypes with human cell lines.

within a host cell. Accordingly, the transgene sequence may be a replicon sequence that directs replication of the vector within the host cell, resulting in an autonomously replicating vector. Alternatively, the transgene sequence may permit vector replication that is dependent upon the host cell's replication machinery.

The base vector sequences may be a operatively linked to a transgene sequence that encodes a gene product, such as a polypeptide, rRNA, or tRNA. For example, the transgene may encode a polypeptide such as a viral capsid protein, or a viral fiber protein. The transgene may be derived from the same or different serotype as the base vector sequence.

Another example of a transgene includes a reporter gene that encodes a gene product that can be used as a selectable marker, such as drug resistance or a colorimetric marker. The reporter gene may encode a gene product which can be readily detected by, for example, a visual microscopic, immunochemical, or enzymatic assay. The preferred reporter gene encodes a gene product that can be detected by a non-destructive method that does not destroy the cell that expresses the reporter gene.

A therapeutic gene is another example of a transgene. A therapeutic gene encodes a gene product (e.g., polypeptide or RNA) which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or the tissue or the organ or the organism containing the host cell. The therapeutic benefit may result from modifying a functin of a gene in the host genome or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The base vector sequence may be linked to a transgene sequence that is an regulatory element, such as a promoters, enhancers, transcription termination signals, polyadenylation sequences. The regulatory element may direct expression of the transgene sequence that encodes a gene product by direct transcription or translation. The regulatory element may regulate the amount or timing of expression of the transgene

sequence. The regulatory element may direct expression of the transgene in certain host cells or tissues (e.g., host-specific or tissue-specific expression).

The base vector sequence may linked to a transgene sequence that permits the vector, to integrate into another nucleotide sequence. The integration sequence may direct integration of the whole vector or portions of the vector. The integration sequence may or may not be related to the base vector sequence. For example the integration and base vector sequences may be from the same or different viral serotype. The integration sequence may be inverted repeat sequences (ITRs) from adenovirus (Ad), adenovirus-associated virus (AAV), or HIV.

The base vector sequence may be linked to a transgene sequence that directs homologous recombination of the vector into the genome of a host cell. Such transgene sequences may or may not be from the same viral serotype as the base vector sequence.

The vector may be used to transport the heterologous sequence into a host cell or into a host cell's genome.

The vector may comprise multiple endonuclease restriction sites that enable convenient insertion of exogenous DNA sequences.

The term "hybrid vector" as used in the invention refers to a vector which comprises a nucleic acid sequence combined from two different viruses (e.g. Adenovirus and AAV).

"Chimeric vector" refers to a vector which contains nucleic acid sequences that are unnatural to the base vector (i.e. sequences not occurring naturally or sequences not in their natural background including heterologous sequences). A chimeric vector as used in the invention may also be a hybrid vector. An example of a chimeric vector is Ad.AAV expressing a modified fiber protein an its capsid.

The term "transduction" or "infection" refers to a method of introducing viral DNA within a virus particle into a host cell. The viral DNA herein is in the form of recombinant virus, which is generated by linking a segment of DNA of interest into the viral genome in such a way that the gene can be expressed as a functional protein.

The term "transfection" refers to a method of introducing a DNA fragment into a host cell.

The term "heterologous" as used herein means that a nucleic acid or peptide sequence is placed in a context that is not endogenous to the base adenovirus vector or to a transduced cell. For example, a peptide sequence can be transferred from a protein to another protein, the resulting protein is referred to herein as heterologous protein. A chimeric fiber protein, (e.g., a serotype 5 tail domain and a serotype 35 shaft and knob domain) is considered a "heterologous" to the Ad 5 vector. The term also includes nucleic acids (e.g. coding sequences) from one strain or serotype of adenovirus introduced into a different strain or serotype of adenovirus.

The term "regulatory elements" is intended to include promoters, enhancers, transcription termination signals, polyadenylation sequences, and other expression control sequences. Regulatory elements referred to in the invention include but are not limited to, those which direct expression of nucleic acid sequence only in certain host cells (e.g. tissue specific regulatory sequences).

The term "operably linked" indicates that a polynucleotide sequence (e.g. a coding sequence or gene) is linked to a regulatory element in such a way that the regulatory element sequence controls and regulates the transcription or translation or both of that polynucleotide sequence. The orientation of the regulatory element may vary (eg, be in reverse orientation with respect to the right ITR). The term also includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence and

production of the desired polypeptide or protein. Regulatory sequences can also include 3' sequences which ensure correct termination (eg. polyadenylation stop signal).

The term "gene therapy" used herein, refers to a method which introduces a segment of exogenous nucleic acid into a cell in such a way that it results in functional modification to the recipient cell by expression of the exogenous nucleic acid. The exogenous nucleic acid is typically therapeutic in that the expression of the encoded protein, polypeptide or RNA corrects cellular dysfunction due to a genetic error or more generally counteracts any undesirable functions which are associated with a genetic or acquired disease. The term "exogenous nucleic acid" refers to DNA or RNA sequences not normally expressed in the treated transformed cell. The term also refers to DNA and RNA sequences which are expressed in a treated transformed cell at a higher, lower or in an otherwise different pattern than in the untreated, nontransformed cell. This non-natural expression can also be termed heterologous expression.

A "gene therapy vector" refers to a vector used for gene therapy. i.e. to introduce the exogenous nucleic acid into a recipient or host cell. The exogenous nucleic acid may be transiently expressed or integrated and stably expressed in the recipient or host cell.

The term "plasmid" as used herein refers to any nucleic acid molecule which replicates independently of the host, maintains a high copy number, and which can be used as a cloning tool.

The term “parallel strand of DNA” and “anti-parallel strand of DNA” refers to as each of the strands of DNA of the double stranded adenovirus. The Figures diagram the location of certain nucleotides on the parallel strand of DNA. The anti-parallel strand of DNA refers to the other of the two strands of DNA which is not depicted in the Figures. The fiber protein is encoded on the anti-parallel strand of DNA. To simplify the vector diagrams, the fiber sequences are shown on the parallel strand even though the gene is located on the anti-parallel strand.

The term "reporter gene" refers to any nucleic acid sequence which encodes a polypeptide or protein which can be readily detected by, for example, a visual, microscopic, immunochemical or enzymatic assay. Preferred reporter genes are those that can be detected by a non-destructive method that does not destroy the treated, transformed cells or tissue.

The term "selection gene" used herein refers to any nucleic acid fragment which encodes a polypeptide or protein whose expression is used to mark a cell as a transformed cell by a given vector.

The term "therapeutic gene" refers herein to a DNA fragment encoding a functional polypeptide, protein or RNA, which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or to the organ or organism containing the host cell. The therapeutic benefit may result from modification of a function of a native gene in a host or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The term "host tissue" or "host cell" as used herein, refers to a tissue or cell in which a therapeutic gene is to be expressed to modify its function.

It is well-known in the biological arts that certain amino acid substitutions may be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate, and isoleucine and valine, are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Volume 5, Supplement 3, Chapter 22, pp. 345-352, which is incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff et

al's frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources. Therefore, any obvious changes in the amino acid sequences (as described above) to the sequences of the invention are already contemplated.

Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups include but are not limited to: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, asparagine-glutamine, and aspartate-glutamate. Therefore, polypeptide substitution for "substantially similar" sequences (as described above) to the amino acid sequences described invention are already contemplated.

In order that the invention herein described may be more fully understood, the following description is set forth.

The present invention provides unique gene transfer vehicles which overcome many of the limitations of prior art vectors. The invention describes a first generation adenovirus vectors comprising left and right Ad ITRs, an Ad packaging sequence, a transgene cassette with regulatory elements, and a pair of cassette ITRs flanking the transgene cassette that direct predictable viral genomic rearrangements during viral replication as well as direct the integration of the transgene cassette into the host cell genome.

One predictable rearrangement that occurs during viral replication is the generation of a gutless adenovirus vector (also referred to herein as ΔAd) that comprises right and left Ad ITRs, an Ad packaging sequence, a transgene cassette flanked by cassette ITRs and the gutless vector is devoid of all other immunogenic viral genes.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the transgene cassette is directed by co-infection with an Ad vector expressing e.g., the rep 78 protein to achieve site-specific integration in the e.g., AAVS1 site on human chromosome 19.

The invention further describes a novel way of targeting these recombinant adenovirus vectors to selected cells by modifying the adenovirus fiber protein that is expressed on the capsid. Changes to both the fiber shaft and the fiber knob domain proved to successfully retarget the Ad vector to a desired cell type. In addition, the G-H loop within the fiber knob domain is identified as a novel site that affect the binding affinity and specificity of the recombinant adenovirus vector. Substitution of peptide sequences into the G-H loop retarget the gutless vector to a desired cell type.

An adenovirus display library has been generated that expresses random peptides within the G-H loop of the fiber protein. This type of a library is used as ligands to screen for adenovirus vectors that bind to desired cell types. One advantage of using an adenovirus display library versus a phage display library is that once adenovirus affinity to a desired cell is identified the targeted adenovirus vector is ready to accept a transgene cassette and can be used to generate a gutless adenovirus vector, for example for use in gene therapy.

The chimeric Ad vectors described below contain a modified fiber protein in the capsid of the adenovirus which renders the vector capable of infecting a desired cell types. Therefore, according to the invention, a gutless chimeric ΔAd-AAV vector can be generated to introduce any transgene(s) into any host cell or tissue which is normally refractory to most commonly used gene therapy viral vectors. In addition, the chimeric ΔAd.AAV vector of the invention, is devoid of adenoviral genes, and contains AAV ITR

sequences that flank the transgene cassette, which direct stable transgene integration in the host genome allowing long term expression of the transgene.

The transgene cassette described in the invention may carry a transgene which is either a reporter gene, a selectable gene for in vitro or in vivo selection of transduced cells, or a therapeutic gene. In one embodiment of the invention the reporter trangene can be but is not limited to, βgalactosidase. Many reporter genes are commonly used in the art, of which any could be carried as a transgene in the Ad.AAV vector of the invention. Other examples of reporter are genes are GFP and alkaline phosphatase.

The following describes an embodiment of the first generation Ad vectors of the invention having a wild-type capsid and a transgene cassette flanked by cassette ITR sequences; (b) fiber protein that is modified to retarget Ad vectors; and (c) the combination of both technologies that enables the production of chimeric ΔAd vector including a modified fiber protein expressed on the capsid which retargets the base vector to a desired cell type for infection and transgene integration.

A. Integrating Ad hybrid vectors of the invention:

It has been shown that inverted repeats (IRs) inserted into the E1 region of AdE1- vectors can mediate predictable genomic rearrangements resulting in a gutless vector genome devoid of all viral genes. A specific embodiment of such IR-mediated rearrangements is the Adeno-AAV, first generation hybrid adenovirus vector containing AAV inverted terminal repeats (ITR) flanking a transgene cassette. The AAV ITRs mediate the formation of a genome similar to that of the ΔAd.IR genome (Steinwaerder et al., 2000 Journal of Virology). ΔAd vectors devoid of all viral genes stably integrate and transduce cultured cells with efficiencies comparable to e.g. rAAV vectors. The Examples demonstrate by Southern blot analysis that the ΔAd vectors integrate randomly into the host genome.

The Ad vectors of the invention comprise a left Ad ITR, an adenovirus packaging sequence located 3' to the Ad ITR; a transgene cassette located 3' to the packaging

sequence comprising a polyadenylation signal, a transgene, and a heterologous promoter, and flanked by a pair of cassette ITRs. Adenoviral genes used for replication such as E1, E2, E3, E4 are located 3' to the right cassette ITR and a right Ad ITR is located 3' to the replication genes. The vectors of the invention are particularly suited to treat: genetic disorders, cancers, and infectious diseases (such as HIV, emboli, or malaria). Treatable genetic diseases such as hemophilia A and B; cystic fibrosis; muscular dystrophy, and $\alpha_1$antitrypsin disorder are ideal candidates for genetic disease that can be treated by vectors of the invention. A specific example of a therapeutic gene to combat a genetic disorder is gamma-globin to ameliorate sickle cell anemia.

To aid in the selection of transduced cellls and characterize the intergration site of the transgene cassette, an embodiment of the invention includes the addition of a sequence comprising a bacterial for the origin of replication, plus a selectable gene. An embodiment of this is an SNori sequence added to the transgene cassette. This allows the ΔAd to be expressed in human and bacterial cells, therefore allowing selection of the transduced cells and characterization of the integration site in the genome of transduced mammalian cells.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein in 293 cells to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

Integration of the transgene cassette contained in the adenoviral vectors into chromosomes may be associated with silencing (or blocking) of transgene expression. The silencing of transgenes can be overcome by adding insulator elements to the transgene cassette. For example, HS-4 insulator elements derived from the chicken-globin LCR can function in Ad vectors to shield heterologous promoters from adenoviral enhancers. HS-4 insulators or the *Drosophila* Gypsy gene can also be used to prevent silencing transgenes.

Another embodiment of the invention is to split the transgene cassette into two portions of the transgene each carried in a different recombinant adenoviral vector of the invention. Each portion of the same trangene has an overlapping region of homology. After infection with both vectors, each carrying the different but overlapping portion of the same transgene, homologous recombination event occurs resulting in the reconstitution of the complete transgene which is then expressed. This technique is used to produce hybrid adenoviral vectors that accommodate large inserts including, but not limited to a 13kb genomic hAAT gene or a 12kb $\tilde{\gamma}$globin LCR$\tilde{\gamma}$globin expression cassettes for ameliorating sickle cell anemia (or correcting γ-globin mutations). The formation of the hybrid ΔAd vector genomes, after recombination between two vectors, is more efficient if the overlapping region of homology within the transgene is longer.

An advantage of the present invention is a method to rapidly isolate pure gutless hybrid adenoviral vectors such as ΔAd.AAV or ΔAd.AAV^{fx} vectors. To minimize the contamination of ΔAd with first generation vectors (Ad vectors) a strategy is described in Example I H. It is anticipated that these approaches will yield the same titer of ΔAd vectors, however the contamination with full-length genome vectors will be less. This improved isolation of the vectors is extremely important to avoid toxic side effects after in vivo application.

B. Tropism modified adenovirus vectors:

The Ad vectors of the invention can be modified so that they target a host cell of interest. There are more than 50 human Ad serotypes (Appendix I), including variants with different tissue selectivity or tropism. It is accepted in the art that different Ad serotypes bind to different cellular receptors and use different entry mechanisms. Most recombinant adenovirus vectors use adenovirus serotype 5 as the base vector serotype 5 (Ad5) (Hitt, M.M., et all, 1997, Adv. in Pharmacology 40, 137-205). Ad5 infection is primarily mediated by its fiber protein binding to CAR and secondarily by its penton base protein binding to integrin. Due to the lack of CAR and/or integrin expression on many cell and tissue types, Ad5 mediated gene transfer is inefficient in a number of tissues which are important targets for gene therapy such as endothelia, smooth muscle, skin epithelia, differentiated airway epithelia, brain tissue, peripheral blood cells, or bone marrow. The following describes Ad5 vectors of the invention having a change in infectivity and tropism as a result of altering the fiber protein sequence.

The infectivity of different Ad serotypes is limited to a number of human cell lines. Infectivity studies revealed that Ad5 and Ad3 are particularly suitable for infecting and targeting endothelial or lymphoid cells, whereas Ad9, Ad11 and Ad35 efficiently infected human bone marrow cells. Therefore, the knob domain of the fiber protein of Ad9, Ad11 and Ad35 are excellent candidates for retargeting the Ad5 vector to human bone marrow cells. Other possible serotypes include Ad7.

In the modified fiber protein of the invention the fiber knob domain of the Ad5 fiber has been replaced with another Ad serotype fiber knob domain. An embodiment of the invention is the modified Ad5/35 fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising of a fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad35). The Ad5/35 chimeric fiber protein shows a broader spectrum of infection to a subset of CD34+ cells, including those with stem cell activity. The Ad5/11 chimeric fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising the fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad11) showed similar tropism.

In addition to the knob domain modifications, the invention describes the added advantage of modifying both the fiber shaft domain and the fiber knob domain to produce a shortened fiber protein. The length of the fiber shaft domain plays a key role in the host receptors used for viral vector entry into the host cell. To show this Ad5, Ad5/9, and Ad5/35 variants were constructed with long (22β-sheets) and short-shafted (7β-sheets)-shafted fibers. These analyses demonstrated that efficient viral infection involving CAR as the primary receptor for Ad5, Ad5/9 requires a long-shafted fiber protein, whereas the cell entry strategy of Ad5/35 (which binds to an still uncharacterized non-CAR receptor) does not depend on the shaft length (Fig. 3). The modification in both the fiber shaft domain length (between 5 > 10 β-sheets) and the fiber knob domain (from a different Adserotype than the base vector is a novel mode of altering Ad vector tropism.

To broaden the repertoire of cell types that Ad vectors can infect, a specific binding region, the G-H loop, within the knob domain has been newly identified herein to improve binding affinity and specificity. Alteration within this region will redirect the Ad vector to a desired cell type. For example, the invention describes the G-H loop sequence within the fiber protein knob domain, which can be replaced with heterologous peptide ligand sequences without affecting the functionally important tertiary structure of the Ad fiber knob domain, while changing the binding affinity and specificty of the vector (Figs. 6,7). This G-H loop region is exposed on the central part of the knob surface and may be strategically a better site for incorporation of heterologous ligands than the peripheral H-I loop (Krasnykh, V. et al., 1998, *J. Virol.*, 72:1844-52.) of the knob C-terminus (Michael, S. I., et al., 1995, *Gene Ther.*, 2:660-8., Wickham, T. J. et al., 1996, *Nat. Biotechnol.*, 14:1570-3.), which are the substitution sites used by others. Therefore, these G-H loop modifications within the fiber knob domain will allow the Ad vector to be redirected to infect a desired cell type, as long as the G-H loop ligand sequence binds to at least one surface protein on the desired cell type. Fig. 7 shows some possible substitutions. Example II J demonstrates that the virion tolerates the insertion of a cycling peptide (12 amino acids) with a constrained secondary structure that allows the exposure on the knob surface. A defined ligand (RGD) can be inserted into the G-H and the H-I loop of an

Ad5 capsid that is ablated for CAR, and integrin tropism. Infectivity studies show the potential advantage of this new insertion site.

Use of the Vectors of the Invention for "Gene Therapy"

The liver is the major organ for protein synthesis. Thereforean important goal of gene therapy is to target gene therapy vectors to the liver. To genetically correct many types of mutant proteins hepatocytes need to be infected with gene therapy vectors carrying a corrected transgene. Example II J describes a G-H loop substitution in the knob domain of the fiber protein with both RI and RII+ (of the malaria circumsporozite surface protein) in a short shafted fiber protein which directs the vector to have affinity and specificity to hepatocytes.

Example II K applies a similar protocol to alter the fiber knob domain in the G-H loop region with peptides that target the vector breast cancer cell lines (MDA-MB-435). These novel approaches to redirect vectors described in the invention allow lower doses of the gene therapy vectors to be administered with a higher safety profile.

In example II L a protocol for preparing an adenovirus display library is described that uses the fiber knob protein to display a library of random peptide sequences within the G-H loop. This library of adenoviruses with modified fiber proteins is screened for affinity and specificity for a desired cell type. There are two main advantages of using this adenovirus display library to screen for target peptides that allow binding to a desired cell type over a phage display library system. First, once a ligand peptide is identified that binds to the desired cell type it is already in the vector of choice for gene therapy delivery. The peptide does not need to be engineered into another vector, as is the case for the phage display library vectors. This reduces the steps required to identify a targeted fiber protein for a desired cell type. The second advantage of this method, is that the adenoviruses are able to display multiple copies of the modified fiber protein on their capsid. This allows for dimerization and trimerization of the fiber protein with the host cell receptor. The multimerization of fibers proteins is a realistic, in vivo interaction of

the trimeric fiber protein with the host cell receptor. In contrast, phage vectors can only display one fiber peptide sequence on their surface, which significantly limits the ability of interaction with host cell surface receptors.

C. A chimeric adenovirus vector with selective tropism:

The chimeric vectors of the invention combine two vectors: an Ad.ITR and a Ad. fx where fx describes a modified fiber protein. A first generation adenovirus vector of serotype 5 is the base vector that carries a transgene cassette flanked by heterologous ITRs. These specific inverted terminal repeat sequences, such as AAV ITRs direct stable integration of the transgene cassette into the host genome as well as control predictable genomic rearrangements that occur during viral replication. This vector can also carry a modified fiber gene (described in Examples II). During replication predictable genomic rearrangements occur which result in the generation of a gutless adenovirus vector (e.g. ΔAd.AAV^{fx}) which expresses the modified fiber protein on its capsid. The modified fiber protein allows the gutless vector to be targeted to a selected cell type. The targeted vector is a gutless adenovirus vector devoid of adenoviral genes which can integrate its transgene into the host genome. The transgene cassette can carry reporter, selectable, or therapeutic genes.

In one embodiment of the invention, the gutless targeted ΔAd.AAV^{fx} carries the reporter gene of βgalactosidase. ΔAd.AAV^{fx} ¯BG). For easy in vitro selection of human and bacterial cells that are transduced with the hybrid Ad vector, a bacterial sequence for the origin of replication can be added to the hybrid Ad vectors. An example of this is ΔAd.AAV^{fx} -Snori, in which a SNori sequence is added into the transgene cassette. This site allows for G418 selection on cells infected with ΔAd.AAV^{fx} –SNori. This in vitro selection provides a tool to analyze the site of transgene integration and the flanking chromosomal regions. Fluorescent in situ hybridization (FISH) is an alternative method to confirm vector integration.

An advantage of the ΔAd chimeric vector for gene transfer is the efficient and stable integration of a large transgene cassette up to e.g. 22kb which is significantly larger than the capacity of retroviral vectors . This is of particular interest for gene therapy. For example, to ameliorate sickle cell anemia ΔAd.AAV^{fx} $\bar{\gamma}$globin , an expression transgene cassette with the gamma-globin gene that targets and integrates, can be inserted into bone marrow stem cells for long term expression of the gamma-globin gene.

To achieve site-specific gene integration, rep78 protein is used for transgene integration into the AAVS1 site (described in Example 1 D). However, this may silence transgene expression. To prevent the integrated transgene from being silenced by host genomic elements (such as positional effects or downstream enhancers), LCRs or insulator elements are incorporated into the transgene cassette.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE I

NOVEL ADENOVIRAL VECTOR Ad.AAV

A. Integrating ΔAd.AAV hybrid vectors devoid of all adenoviral genes.

In vitro and *in vivo* studies with rAAV indicate that the only requirement for rAAV integration are the AAV ITRs and as yet unknown host cellular factors. It is thought that specific sequences or secondary structures present in AAV ITRs are prone to integration into host chromosomal DNA. In order to combine advantages of adenoviral vectors (high titer, high infectivity, large capacity) and the integration capability of AAV ITRs, AAV vector DNA with AAV ITRs flanking cassettes a secreted human placental alkaline phosphatase (SEAP) - neomycin phosphotransferase (neo) reporter gene cassette

(Alexander, I.E., et al. 1996, *Gene Therapy*, 7, 841-850) is incorporated into the E1-region of E1/E3 deleted adenoviral vectors (Ad.AAV1) (Figure 1, top).

METHODS

Production/Characterization of Viral Vectors

Plasmids:

The AAV1 vector cassette containing AAV ITRs and SEAP/neo expression units is obtained by AseI/ScaI digestion of the plasmid pALSAPSN (Alexander, I. E. et al, 1996, *Human Gene Therapy*, 7:841-50). The 4.4kb AAV vector fragment was cloned via NotI adapter linkers into pXJCL1 (Mirobix, Toronto, Canada) (pAd.AAV1). Another shuttle vector (pAd.AAV1-Δ2ITRs) lacking the AAV ITRs is generated by inserting the 3.7kb AflII/BsmI fragment of pALAPSN into pXJCL1. For pAd.AAV1Δ1ITR, a construct is used where a spontaneous deletion in the left AAV ITRs between the A and A' regions has occurred. To create a second hybrid vector (Ad.AAV2), the AAVSNori cassette developed by E. Rutledge is used. AAV vector DNA obtained is from pASNori (Rutledge, E. A., Russell, D.W. 1997. *Journal of Virology* 71:8429-8436) as a 3.4kb BsaI/ScaI fragment and inserted into the EcoRV site of pXCJL1. As it is generally known for AAV vector plasmids, the AAV ITRs are prone to rearrangements. To minimize deletions in these functional critical regions, all constructs for generation of hybrid vectors are assembled in low copy-number plasmids which are grown in E. coli Top10, JC811, or XL1 Bluecells (Stragene, La Jolla, Calif.). Furthermore, after each cloning step or large-scale plasmid amplification, both AAV ITRs are carefully mapped by restriction analysis with enzymes that cut inside or adjacent to the ITRs (BssHII, AhdI, SmaI, BglI, BsmI, AflII, and ScaI).

Adenoviruses:

First-generation viruses with the different transgene cassettes incorporated into the E1 region are generated by recombination of the pΔE1aSpla- or pXCJL1-derived shuttle plasmids and pJM17 (Microbix) in 293 cells as described earlier (Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791). For each virus, at least 20 plaques are picked, amplified, and analyzed by restriction digest. Viruses containing two AAV ITDRs tend to rearrange within the ITRs, with other adenoviral sequences, or with adenoviral sequences present in the 293 cell genome. Only plaques from viruses with intact ITRs are amplified, CsCl banded, and titered as described earlier (Kay, M. A., et al. 1995. *Hepatology* 21:815-819; Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960). All virus preparations tested are negative for RCA and bacterial endotoxin (Lieber, A., et al.1997. *Journal of Virology* 71:8798-8807). Virus is stored at –80°C in 10mM Tris-Cl, pH 7.5-1 mM $MgCl_2$-10% glycerol.

To generate ΔAd.AAV, 293 cells are infected with Ad.AAV1 at an multiplicity of infection (MOI) of 25 and harvested 40h after infection. Cells are lysed in PBS by 4 cycles of freeze/thawing. Lysates are centrifuged to remove cell debris and digested for 30 min at 37°C with 500 units/ml DNaseI and 200 μg/ml RNaseA in the presence of 10mM $MgCl_2$. 5ml of lysate is layered on a CsCl step gradient (0.5ml - 1.5g/cm^3, 2.5ml - 1.35g/cm^3, 4ml - 1.25g/cm^3) and ultracentrifuged for 2h at 35,000 rpm (rotor SW41). CsCl fractions are collected by puncturing the tube and are analyzed for viral DNA (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960; Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) or subjected to ultracentrifugation at 35,000 rpm for 18 hours in an equilibrium gradient with 1.32 g/cm^3 CsCl. The band containing the deleted viruses ΔAd.AAV is clearly separated (0.5cm distance) from other banded viral particles containing full-length ad.AAV genomes. ΔAd.AAV1 fractions are dialyzed against 10mM Tris-Cl, pH 7.5-1mM $MgCl_2$-10% glycerol and stored at –80°C. The genome titer of ΔAd.AAV1 preparations is determined based on quantitative Southern analysis of viral DNA purified from viral particles in comparison to different concentrations of a 4.4kb AseI/ScaI fragment of pALSAPSN according to a protocol described earlier (Lieber, A.,

et al., 1996, *J. of Virology*, 70, 8782-8791). In total, the production of 1×10^{13} genome-particles of ΔAd.AAV1 requires less than 3 hours of actual work.

Titers routinely obtained are in the range of $3\text{-}8 \times 10^{12}$ genomes per ml. Assuming one genome is packaged per capsid, the genome titer equals the particle titer. The level of contaminating Ad.AAV1 is less than 0.1% as determined by Southern analysis, which is consistent with results obtained by plaque assay on 293 cells (fewer than 5 plaques per 10^6 total genomes). The primers used for sequencing the left and right ITR-vector-junction are

5'GGCGTTACTTAAGCTAGAGCTTATCTG, and
5'CTCTCTAGTTCTAGCCTCGATCTCAC.

The recombinant AAV virus stock containing the SEAP/neo cassette (AV2/ALSAPSN, [Alexander, I. E. et al, 1996, *Human Gene Therapy*, 7:841-50] used in these studies were obtained from Dusty Miller (FHCRC, Seattle). The stock was free of contaminating replication competent AAV (<50 particles/ml) and wildtype adenovirus (<100 particles/ml). The genome titer of the virus stock was obtained by quantitative Southern Blots as described by Russell et al. (Russell, D. et al. 1994 *Proc. Natl.Acad,Sci. USA* 91:8915-8919).

Electron Microscopy:

For examination of viral particles in the transmission electron microscopy studies, CsCl-purified virions are fixed with glutaraldehyde and stained with uranyl acetate as described previously (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960).

RESULTS

During replication of these hybrid vectors in 293 cells, a 5.5kb genome (ΔAd.AAV1) is efficiently generated and packaged into adenovirus (Ad5) capsids. The ΔAd.AAV1

genome contains the left adenovirus ITR and the packaging signal followed by the AAV-vector cassette and a duplicate of the adenoviral packaging signal and ITR in reverse orientation (Figure 1, bottom). The hybrid vector is devoid of all viral genes, thus eliminating toxic effects and the elicitation of cellular immune responses. The spontaneous formation of the small hybrid vector genome ΔAd.AAV1 requires the presence of two intact AAV ITRs and does not occur with partly deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette.

Hybrid vectors containing different transgenes:

To construct a hybrid vector with a transgene that can be detected in situ the SEAP/neo expression unit in Ad.AAV1 is replaced by the *E. coli* β-galactosidase gene. This hybrid vector is named ΔAd.AAV1. During generation of the corresponding plasmid constructs the AAV ITR sequences tend to rearrange and abolish their functional properties. This problem can be circumvented by using low copy number plasmids as cloning vectors grown in bacteria strains depleted for all recombination proteins (e.g.JC811). Furthermore, the intactness of both AAV ITRs after each cloning step can be examined for characteristic endonuclease digestion. Recently, another hybrid vector ΔAd.AAV1Nori has been generated which contains the neo gene under the control of both the simian virus 40 (SV40) early promoter and the transposon 5 (Tn5) promoter for expression in human and bacterial cells, as well as the p15A bacterial replication origin with the direction of the leading strand DNA synthesis opposite that of neo gene transcription. Thus, SNori can be used for G418 selection of integrated vector in eukaryotic cells as well as for rescue of vector together with flanking host DNA after integration. The recovered plasmids can be propagated in *E. coli* under selection with kanamycin due to the bacterial origin and the neo gene. SNori containing vectors allow a rapid estimation of total integration events based on the number of G418 resistant colonies. Moreover, vector DNA together with flanking chromosomal DNA can be rescued as plasmids from single G418 resistant clones and can be used for sequencing to determine integration junctions. Both hybrid vectors are produced at a titer of about

$3x10^{12}$ genomes per ml. The ratio of genome titer to transducing particles for ΔAd.AAVBG is ~200:1 based on β-Gal expression.

DISCUSSION

ΔAd.AAV1 could spontaneously form during adenovirus replication. Another possible mechanism of ΔAd.AAV1 formation is based on the unique mechanism by which adenovirus replicates its genome (van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin) (see Figure 1). Ad DNA replication is initiated by the TP/pTP (terminal protein) that binds to specific sites within the ITRs on both ends of the linear genome and catalyzes, in complex with Ad pol, the binding of the 5' CTP, the first nucleotide of the daughter strand. DNA synthesis proceeds in a continuous fashion to the other end of the genome (Figure 1A). Only one of the DNA strands serves as template. One of the replication products is a single-stranded DNA that circularizes through annealing of its self-complementary ITRs. The resulting duplex "panhandle" has the same structure as the termini of the duplex viral genome that allows the binding of pTP and the initiation for synthesis of a complementary strand using the single-stranded "pandhandle" molecule as template (Figure 1C). In the case of Ad.AAV1, the Ad pol synthesizes the single strand of the adenoviral genome starting from the left Ad ITR until it reaches the second AAV ITR. During synthesis of the second AAV ITR a certain percentage of the single-stranded molecules form a loop hybridizing to the complementary region within the first AAV ITR that was replicated earlier, allowing Ad pol to use the same viral DNA strand to read back towards the left ITR (Figure 1B). The resulting "panhandle" structure can be resolved in a similar way as a full-length intermediate shown in Figure 1C, generating a double stranded, linear molecule with the above described structure that can be packaged into Ad virions. The ratio of viral DNA to protein concentration in purified ΔAd.AAV1 particles is comparable to that obtained from Ad.AAV1 particles. This indicates that despite the smaller size, only one ΔAd.AAV1 genome is packaged, resulting in particles with a lighter buoyant density (~1.32 g/cm^3). Electron microscopy demonstrates the icosahedral shape of ΔAd.AAV1 particles (Figure 2). Staining with uranyl acetate causes the central viral cores to appear

electron dense. ΔAd.AAV1 virions have only a spotted luminal dark staining as expected with only one 5.5kb genome being packaged per capsid.

B. *In vitro* ΔAd.AAV1 production:

Characteristics of deleted adeno-AAV vectors (ΔAd.AAV):

A number of experiments to clarify the mechanisms of ΔAd.AAV genome formation are carried out. Specifically, the presence of two intact AAV ITRs flanking a reporter gene cassette is required for the effective formation of ΔAd.AAV genomes. This process does not occur with partially deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette. Furthermore, in vitro transduction studies are performed with different genome titers of ΔAd.AAV1, Ad.AAV1, and Ad.AAV1-Δ2ITRs, (lacking the two AAV ITRs) which determine the number of G418 resistant colonies that formed after 4 weeks of selection (Table I).

ΔAd.AAV1 is routinely produced at a high titer (5×10^{12} genomes per ml with $>10^4$ produced genomes per 293 cell) and at a high purity with less than 0.1% contaminating full length Ad.AAV1 genomes by a technique normally used for amplification and purification of recombinant adenovirus.

In vitro transduction studies with hybrid vectors on CD34+ cells and erythroleukemia cells:

In order to test whether the hybrid vectors allow for gene transfer into cell types, that have to be targeted for sickle cell therapy, infection/transduction studies are performed using CD34+ enriched human bone marrow cells, derived from mobilized peripheral blood and the human erythroleukemia cell line K562 which express ε and γ globin genes.

METHODS

Cell Culture:

SKHep1 cells (HTB-52, American Type Culture Collection, Rockville, MD), an endothelial cell line derived from human liver [Heffelfinger, S.C., et al., 1992, *In vitro Cell Dev. Biol.* 28A, 136-4-142], are grown in high-glucose Dulbecco's modified Eagle medium with 10% fetal calf serum. SKHep1 cells are analyzed for integrated AAV provirus by Southern analysis of genomic DNA using the AAV1 wild type genome obtained from pAAV/Ad (Samulski, R. J., et al. 1989. *Journal of Virology* 63:3822-3928) (gift from David Russell, University of Washington) as a probe. No specific bands are detected in undigested genomic SKHep1 DNA or after digestion with HindIII. For viral infection, confluent cells are incubated with different viral doses for 2 hours, followed by intensive washing. For G418 selection, 24h after infection with ΔAd.AAV1, SKHep1 cells are trypsinized and plated at different dilutions under G418 selection (900μg/ml active compound, Boehringer-Mannheim, Germany). G418 containing culture medium is changed every 3 days. The number of colonies with $>10^4$ cells is counted after 4 weeks of selection and divided by the number of initially seeded cells. This ratio is used to express the integration frequency of ΔAd.AAV1. Single colones are obtained by limiting dilutions of infected cells in 96 well plates. Colonies are expanded to 1×10^6 cells in the presence of G418. Immunofluorescence analysis for adenoviral proteins expressed in SKHep1 cells 3 days post-infection is performed as described earlier [Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791].

RESULTS

293 cells are infected with the first generation vector Ad.AAV1. During replication of Ad.AAV1, the small ΔAd.AAV1 genome forms spontaneously and is packaged into adenovirus capsid. At 36 hours after infection cells are harvested and virus is released by several cycles of freeze/thawing. The mixture of Ad.AAV1 and ΔAd.AAV1 particles in the cell lysate is then separated by ultracentrifugation in a CsCl step gradient. Due to its lighter buoyant density, the band containing the ΔAd.AAV1 particles is clearly separated (0.8cm distance) from the band containing full-length virus (Lieber, A., et al. 1999. *J*

Virol 73:9314-24). ΔAd.AAV1 is purified further by an additional CsCl equilibrium gradient and is stored in 10mM Tris pH7.5, 10% glycerol, 1mM $MgCl_2$ in 80°C. In total, the production of $2x10^{13}$ (genome) particles of ΔAd.AAV1 requires less than 3 hours of work. All functions for ΔAd.AAV1 replication and particle formation are provided from Ad.AAV1 genomes amplified in the same cell. The efficiency of vector production measured on a genome-per-cell-basis is comparable or higher than labor-intensive, newer techniques for rAAV production, which have not yet been proven to be reliable. The estimated ratio of transducing/genome titer for ΔAd.AAV1 is 1:200 (based on SEAP expression at day 3 post-infection), whereas for the average rAAV preparation, it is in the range of $1:10^3$ to $1:10^4$. $1x10^5$ confluent SKHep1 cells are infected with different MOIs of rAAV1 (stock: $1x10^{10}$ genomes per ml), ΔAd.AAV1 (stock: $5x10^{12}$ genomes per ml), Ad.ADAV1 (stock: $1x10^{13}$ genomes per ml), and AdAAV1.2ITR (stock: $9x10^{12}$ genomes per ml), in a volume of 100ml 24 hours after infection, cells are washed, trypsinized, and plated at different dilutions. G418 is added 24 hours after plating and selection is performed for 4 weeks. G418 resistant colonies contain on average $>5x10^4$ cells (at least 16 cell divisions). A significant number of small colonies visible at 2 weeks post-infection do not survive continued selection, probably due to episomal vector expression. Cells infected with first-generation adenoviruses with MOIs greater than $1x10^4$ develop CPE during the first week of selection. The rAAV titer is not high enough to perform infection studies with MOIs greater than 10^4. The colony formation is expressed as percentage of the number of colonies after selection to the number of cells initially seeded for selection (Table I).

TABLE I

Formation of G418 resistant colonies after infection with hybrid viruses in comparison with rAAV.

MOI (genomes per cell)	Formation of G418 resistant colonies in % (SEM) (after 4 weeks of selection)

	rAAV1	.Ad.AAV1	Ad.AAV1	Ad.AAV1 .2ITRs
10^1	0	0	0	0
10^2	0	0	0	0
10^3	2.7 (1.6)	1.3 (1)	5.4 (3.0)	0
10^4	90.8 (7.0)	48.0 (8.9)	12.9 (7.2)	0
10^5	N/A	93.1 (5.4)	3.8 (2.1)	0
10^6	N/A	100	0	0
10^7	N/A	100	0	0

N = 3 (SEM is indicated in parentheses.)

K562 cells are infected with different MOIs of ΔAd.AAVBG (1-10^8 genomes per cell):

Three days after infection, the total number of viable cells (based on Trypan blue staining) and the percentage of infected cells (based on X-Gal staining) are determined for all MOIs. The results are presented in Figure 4A.

Initial integration studies:

K562 cells are incubated with ΔAd.AAVSNori at an MOI of $2x10^5$ genomes per cell and the colonies that formed after 4 weeks of G418 selection are counted in 96 well plates. G418 resistant colonies contain on average >$5x10^4$ cells which means that the original cell underwent at least 16 cell divisions.

Infection studies with .Ad.AAVBG (1-10^8 genomes per cell) on CD34+cells:

Cell infection on CD34+are as described for K562 cells. CD34+ cells are cultured in IMDM supplemented with 20% FCS, kit ligand (stem cell factor-SCF) (100ng/ml), and IL-3 (100ng/ml). Since a number of reports suggest that specific cytokines like GM-CSF or M-CSF which induce stem cell differentiation can stimulate integrin expression and may therefore affect internalization of Ad5 vectors, infection rates are compared with

Ad5 based hybrid vectors on CD34+ cells cultured with and without pre-stimulation with GM-CSF (50ng/ml) or M-CSF (50U/ml). The number of infected cells is counted based on X-Gal staining at day 3 after infection. To test for dose-dependent toxicity, viable cells are counted (based on trypan blue exclusion) at day 3 post-infection. Furthermore, whether high viral doses affect the ability of CD34+ cells to differentiate in methyl cellulose colony assays in presence of IL-3 and SCF is analyzed. The results are expressed as viable cells/X-Gal positive cells vs MOI (see Figure 4B).

DISCUSSION

The above data demonstrate that ΔAd.AAV transduces stably an immortalized human cell line with a low frequency comparable to rAAV, however, transduction rates could be scaled up to 100% by using greater MOIs of ΔAd.AAV1, which is produced at higher titers than rAAV1. In contrast to infection with the first-generation vector, Ad.AAV1, infection with ΔAd.AAV1 is not associated with dose-dependent cytotoxicity because no viral proteins are expressed from these vectors in transduced cells. Furthermore, viral proteins present in the incoming ΔAd.AAV1 particles are not problematic in the dose range use. The comparison of transduction rates of ΔAd.AAV/Ad.AAV1 with the vector lacking AAV ITRs, Ad.AAV-Δ2ITRs, supports the hypothesis that the presence of two intact AAV ITRs is crucial for hybrid vector integration.

The data demonstrate that the leukemia cell line can be infected at ~90% efficiency with Ad5 based hybrid vectors at MOIs $2x10^5$ genomes per cell without significant toxic side effects. However, this dose is still ~100 times greater than the dose necessary to infect 100% of HeLa cell, hepastoma cells, primary hepatocytes and other cell lines generally considered as permissive for Ad5 vector infection.

Since viral DNA in cells infected with $2x10^4$ genomes (or 100 transducing particles per cell) should be lost after 7 cell divisions, the presence of G418 resistant cells in the observed colonies suggests that ΔAd.AAVSNori genomes are integrated into or stably associated with the host genome. Based on the number of G418 resistant colonies one

out of 25,000 ΔAd.AAVSNori genomes integrates stably into K562 cells. This is in agreement with the results obtained earlier with ΔAd.AAV1 in SK Hep1 cells.

The maximal dose used for infection of CD34+ cells ($1x10^8$) results in X-Gal staining of only ~10% of cells independently of GM-CSF/M-CSF. This demonstrates the obvious inability of Ad5 to infect CD34+ cells and is probably caused by the absence of specific receptors and/or integrins on the cell surface. CD34+ cells tolerate a large range of viral doses ($1\text{-}10^7$) without obvious effects on cell viability and total cell number. This is not surprising because in order to develop toxic side effects adenovirus has to enter the cell and express viral genes. Hybrid vectors can be produced at titers of $5x10^{12}$ genomes per ml. Thus, the maximal MOI that can be used for infection (of 10^4 cells) is ~$5x10^8$ (in 100µl storage buffer). Based on the infection studies with ΔAd.AAVBG this dose may not be sufficient to efficiently transduce CD34+ cells and to obtain an appreciable number of G418 resistant colonies.

C. *In Vivo* Properties Of ΔAd.AAV1:

Viral DNA is labeled with BrdU during virus amplification to investigate cellular/nuclear vector uptake in situ. For transduction studies, confluent SKHep1 cells (a human endothelial cell line) are infected with 2000 genomes ΔAd.AAV1 or Ad.AAV1 per cell. BrdU tagged viral DNA is detected in 100% of nuclei at 3 hours post-infection for both viruses indicating efficient cellular and nuclear uptake of hybrid virus DNA.

RESULTS

The ΔAd.AAV1 vector transduces a cell in vitro forming G418 resistant colonies with an efficiency of 17 or 58%, after infection with an MOI of $1x10^3$ or $1x10^4$ genomes per cell, respectively. Approximately $2x10^4$ ΔAd.AAV1 genomes are required to yield one stable transfectant. Since all stable colonies contain integrated ΔAd.AAV1 vector DNA, this number reflects the minimal integration frequency of ΔAd.AAV1 in SKHep1 cells which is comparable with that from rAAV (Rutledge, E. A. et al., 1997, *Journal of Virology,*

71:8429-36). The number of G418 resistant colonies does not necessarily represent the total frequency of integration events because not all integrated copies express neomycin phosphotransferase, due to chromosomal position effects or incomplete integration.

The absence of adenoviral gene products in ΔAd.AAV1 transduced cells at day 3 post-infection is demonstrated by immunofluorescence with antibodies to the major late proteins (hexon, fiber) and early proteins (DBP.E4-orf6). Expressed adenoviral proteins are detected only in cells infected with Ad.AAV1. The fact that cells infected with ΔAd.AAV1 do not express potentially cytotoxic adenoviral proteins is important.

While an MOI of $1x10^4$ genomes per cell of the first generation vector Ad.AAV1 induce cytopathic effects in SKHep1 cells at day 3 p.i., no toxic side effects are observed when SKHep1 cells are infected with ΔAd.AAV1 at a dose of up to $1x10^8$ genomes per cell. Since the transduction efficiency is clearly dose dependent, ΔAd.AAV1 (which can be produced at titers of >$5x10^{12}$ genomes/ml) is able to stably transduce Ad5 permissive cell lines or tissues with a 100% efficiency without associated toxicity.

Southern analysis indicates that ΔAd.AAV1 integrates randomly as head-to-tail tandem repeats into the host cell genome via the right AAV ITR, whereas the other junction with the chromosomal DNA is variable and occurs somewhere within the transgene cassette. In order to confirm the integrated status of ΔAd.AAV1 DNA, high-molecular-weight chromosomal DNA is separated by pulse field gel electrophoresis (PFGE), followed by Southern analysis with a SEAP specific probe (Figure 3). Undigested DNA from control SKHep1 cells give an endogenous SEAP signal that co-migrates with chromosomal DNA just below the well (lanes 1 and 5). No high-molecular weight episomal forms of ΔAd.AAV1 DNA are detected, whereas a distinct 35 kb band is visible in DNA from SKHep1 cells isolated 3 days after infection with first generation adenovirus, Ad.AAV1 (lanes 4 and 13). Digestion with EcoRI reveals the 4.4kb fragment, which is specific for integrated tandem copies of the AAV cassette (lanes 8 and 12). To eliminate the possibility that chromosomal DNA is trapped in the well, DNA samples are digested with intron-encoded endonucleases PI-SceI or I-CeuI (Gibco-BRL, Grand Island, NY) with a

sequence specificity or more than 11bp or 9bp respectively. Digestion with PI-SceI yields a >2mb endogenous SEAP signal in SKHep1 cells (lane 2) and an additional signal in the range of ~1mb in G418 resistant colonies transduced with ΔAd.AAV1 (lane 7). I-CeuI digestion results in a smear between 250-1000 kb in ΔAd.AAV1 transduced SKHep1-cells (lanes 10, 11) indicating random integration, whereas a high-molecular weight band specific for the endogenous SEAP gene is observed in control SKHep1 cells (lane 9).

One day after intraportal infusion of $1x10^{12}$ ΔAd.AAV1 genomes in C57Bl/6 mice, BrdU labeled vector genomes can be detected in 85% hepatocytes (Lieber, A., et al. 1999. *J Virol* 73:9314-24). Hepatocellular DNA analysis performed at 2 months post-infusion reveals ΔAd.AAV1 DNA integrated with an average of 0.5 copies per cell into the mouse genome (Lieber, A., et al. 1999. *J Virol* 73:9314-24). To assess potential side effects of intraportal ΔAd.AAV1 infusion, serum glutamic pyruvic transaminase (SGPT), a sensitive marker for hepatocellular injury, is measured for 7 consecutive days post-infusion in combination with histological analysis of liver sections. No significant elevation in SGPT levels, or histological abnormalities are detected after intraportal infusion of $1x10^{12}$ or $1x10^{13}$ ΔAd.AAV1 genomes, whereas infusion of the same dose of full-length Ad.AAV1 vector is associated with severe hepatoxicity or fatal outcome. This suggests that the dose of ΔAd.AAV1 administered to mice can be increased to obtain higher transduction efficiencies in vivo without adverse side effects, which is not possible for first generation adenoviruses. Importantly, ΔAd.AAV1 transduced quiescent hepatocytes in vivo, which suggests that integration of hybrid vector DNA may not require cell proliferation. Recently, more detailed in vivo transduction studies with Ad.AAV1 and ΔAd.AAV1 have been performed in Balb/c mice to study whether the absence of adenoviral gene expression in cells infected with ΔAd.AAV1 can avoid an anti-viral immune response and can prolong vector persistence. In this mouse strain, vector DNA is cleared from the liver at 4-6 weeks after infusion with first generation adenoviruses, mostly due to a CTL response against viral proteins produced in transduced cells. Vector DNA is analyzed by genomic Southern Blot of hepatic DNA at 12 weeks

after infusion of $1x10^{12}$ genomes Ad.AAV1 or ΔAd.AAV1. At this time point, no vector specific signal is detectable in hepatic DNA from mice infused with the first generation vector Ad.AAV1, while ~0.3 copies of ΔAd.AAV1 genomes per cell are present in livers of mice that received the hybrid vector, again indicating the superior in vivo properties of the hybrid vector.

D. Effects of Rep Coexpression on ΔAd.AAV Integration

Rep expression after plasmid transfection:

In order to test whether Rep expression enhances site-specific integration of ΔAd.AAV1 in human cells, a series of Rep expression plasmids are constructed.

METHODS

The Rep ORF 68/78 (nt 285-2313) including the internal p19 and p40 promoters is obtained from pAAV/Ad (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia) by digestion with BsaI/BsrI. This fragment deleted for the AAV p5 promoter is cloned via adapter linkers under RSV or PGK promoter in front of the bovine growth hormone polyadenylation signal (bPA) into pAd.RSV or pAd.PGK (Lieber, A., and Kay, M.A., 1996, *J. of Virology*, 70, 3153-3158; Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) correspondingly.

RESULTS

The resulting plasmids (pRSVrep, pPGKrep) are transfected into 293 cells or SKHep1 cells, most of the Rep proteins expressed from the heterologous promoters (RSV or PGK) are Rep 68 and Rep 78, while transfection of the rep gene under aP5 promoter (pAAV/Ad) results in predominant Rep 52/40 expression. Thus, transfection of pRSVrep and pPGKrep is more pronounced suggesting a strong transactivation of AAV promoters

by Ela which is produced in 293 cells. This result indicates that minimum expression of rep proteins is necessary to avoid interference with adenovirus replication.

Rep-mediated site-specific integration of ΔAd.AAV1.

The potential for site-specific integration is an important characteristic of the novel Ad.AAV vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

A preliminary test can be performed to confirm the functional activity of Rep 68/78 expressed from pRSVrep to mediate site-specific integration of ΔAd.AAV1 (Figure 5 and 6). Human SKHep1 cells are transfected with pRSVrep or control plasmid (pRSVbGal (Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) (transfection efficiency was ~20%), followed by infection with ΔAd.AAV (2000 genomes per cell). Three days after infection, cells are trypsinized, embedded in agarose, lysed in situ, digested with I-CeuI (an intron-encoded endonuclease with a recognition sequence of more than 10nt), subjected to pulse file gel electrophoresis in 1% agarose gel, and analyzed by Southern Blot. Hybridization with a probe covering the AAVS1 integration site (1.7kb EcoRI/BamHI fragment from the chromosome 19 locus (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia)) reveals an AAVS1-specific band (~240kb) in I-CeuI digested DNA from cells after control plasmid transfection (pCo) + ΔAd.AAV1 infection. An additional signal in the range of 280kb appears in rep expressing cells infected with ΔAd.AAV1

(pRSVrep + VAd.AAV1) indicating a site-specific insertion into the AAVS1 site in a certain percentage of cells. The presence of vector DNA in this 280kb band is confirmed by rehybridization of the same filter with a transgene (SEAP) specific probe. Randomly integrated ΔAd.AAV1 vector appears as a diffuse SEAP signal in the range 280-680kb (pCo+ΔAd.AAV1, pRSVrep+ΔAd.AAV1). The specific ~1.9 mb band on blots hybridized with the SEAP probe represents an I-CeuI fragment containing the endogenous human SEAP gene.

Incorporation Rep 68/78 function into hybrid vectors to stimulate site-specific integration

Rep overexpression inhibits adenovirus DNA replication, prohibiting the generation of rep expressing Ad vectors using conventional strategies. To solve this problem, significant Rep 68/78 expression from the hybrid vector in virus producer (293) cells must be prevented while maintaining transient Rep expression in target cells (HSC) to mediate site-specific integration. Our hypothesis is that the specific structure of the ΔAd.AAV hybrid virus can be used to bring the rep gene 68/78 into a transcriptionally active position under control of a HSC specific promoter only at late stages of virus replication in 293 cells. This will allow amplification of the hybrid vector in 293 cells, generating high titer virus which activates the incorporated Rep 68/78 functions only in HSC. The general outline of our strategy to produce Rep expressing hybrid vectors is illustrated in Figure 7. The rep/transgene cassette is assembled based on the left-hand shuttle plasmid used for recombinant adenovirus production. The gene encoding Rep 68/78 is cloned in 3'□5' orientation in front of a transgene expression cassette flanked by AAV ITRs. Between the transgene cassette and the right AAV ITR an HSC-specific promoter is inserted with direction towards the adenoviral E2, E3, and E4 genes. The recombinant genome is produced by recombination in E.coli and transfection into 293 cells generates virus (Ad.AAV-rep). The specific structure of ΔAd.AAV with duplicated sequences flanking the AV ITRs is used to bring the rep gene into a transcriptionally active position under control of a HSC specific promoter only during late stages of viral DNA replication in 293 cells. During amplification of Ad.AAV-rep, the smaller genome ΔAd.AAV-rep is formed and packaged into particles, which can be separated by ultracentrifugation in CsCl gradients. The specific structure of ΔAd.AAV-rep brings the

rep gene into 5'↔3' orientation in relation to the HSC specific promoter, allowing rep transcription in target cells. After transduction of HSC with purified ΔAd.AAV-rep particles, rep expression is activated and mediates rescue of the AAV-ITR/transgene cassette from the adenoviral vector backbone and site-specific integration. The hypothesis is that Rep-mediated integration into AAVS1 occurs via the right or both AAV/ITRs causing the rep gene to become separated from the hepatocyte-specific promoter once the vector is integrated (Figure 7). Therefore, rep expression should be only transient without critical cytotoxic side effects on the host cell.

Promoters that can regulate rep expression:

Potential candidate promoters to drive rep expression with high specificity for HSC and minimal activity in 293 cells are the 454nt CD34 promoter (Krause, D.S., et al., 1997, *Experimental Hemotology*, 25, 1051-1061; Yamaguchia, Y. et al., 1997, *Biochimica et Biophysica Acta.*, 1350:141-6), the 300nt HS 40 enhancer (Chen, H.L., et al., 1997, *Nucleic Acids Res.* 25, 2917-2922) or a 3kb CD34 enhancer (May, G. et al., 1995, *EMBO J.*, 14:564-74) in combination with an initiator, or the HIV LTR. An optimal promoter is selected based on studies of transient reporter gene expression after plasmid transfection in 293 cells and hepatocytes. All promoters to be tested are cloned in front of the human α_1-antitrypsin (hAAT)- bovine growth hormone polyadenylation signal (bPA) into the adenoviral shuttle plasmid pCD2 (pAd.-hAAT). Promoter activity can be tested in transient plasmid transfection assays in CD34+ and 293 cells. The promoter with the highest hAAT levels in CD34+ or K562 cells and the lowest hAAT expression in 293 cells is selected for further studies. If high background expression in 293 cells from these promoters is seen, insulators to shield HSC-specific promoters from the Ela enhancer which is still present in Ad shuttle plasmids can be utilized.

Rep genes:

The large Rep 68/78 proteins are sufficient to mediate rescue and site-specific integration. Unregulated Rep 52 and Rep 40 expression from the AAV p19 promoter

located within the ORF of Rep 68 and 78 must be prevented because production of these smaller Rep proteins in 293 cells will affect cell viability and adenoviral DNA synthesis. To do this, constructs obtained from Surosky et al., containing a mutated Rep 52/40 start codon to express Rep 68 and 78 individually under CMV promoter can be used. The 293 cells transiently expressing Rep68 or Rep 78 from these constructs can be coinfected with ΔAd.AAV1 (infection 24 hours after pCMVRep transfection, MOI $2x10^5$ genomes/cell). Three days after ΔAd.AAV1 infection, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE as described earlier. Efficient Rep mediated excision of the AAV cassette and site-specific integration without flanking adenoviral sequences are expected and the plasmids pCMVRep68 or pCMVRep78 can be used as a source for the corresponding rep genes and clone them into hybrid vectors.

Vectors:

The rep/transgene cassette can be assembled based on pXCJL (Microbix, Toronto). A set of control hybrid vectors can be generated with the AAV-ITR-transgene cassette only without the rep gene. The recombinant Ad.AAV-rep genome can be generated by recombination of the left hand shuttle plasmids with pCD1, a pBHG10 (Microbix, Toronto) derivative, which contains the Ad5 genome deleted for the E1/E3 regions in $recA^+$ E. coli (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Compared to the standard technique based on plasmid recombination in 293 cells, this approach has the advantage that plaques with recombinant virus appear 3 times faster and the production of illegitimate recombinants is minimized. This allows efficient viral DNA amplification and packaging to occur before Rep expression reaches levels that are potentially inhibitory for adenoviral replication. The critical variables in maximizing the output of the vector deleted for all adenoviral viral genes are the initial multiplicity of infection and the time of harvesting. These parameters can be optimized for production of ΔAd.AAV-rep hybrid vectors. A number of ΔAd.AAV vectors can be constructed incorporating rep gene. Cryptic promoter and enhancer elements present in the 5'-342nt of the adenoviral genome can interfere with transgene expression from the heterologous promoters. This is crucial for the strategy to avoid rep expression from ΔAd.AAV-rep genomes in 293 cells.

To ensure efficient transgene expression, insulator fragments such as the chicken beta-globin insulator can be used with a selected promoter, constitutive or inducible.

Rep protein co-packaging:

As an alternative to producing hybrid vectors containing the rep 68/78 gene, studies are designed to see whether Rep protein can be co-packaged into ΔAd.AAV capsids and whether these co-packaged Rep molecules are sufficient to mediate rescue and site-specific integration of the AAV-ITR-transgene cassette. Our hypothesis is that the Rep 68/78 binds to the Rep binding site (RBS) present in double-stranded ΔAd.AAV genome and that this complex is co-packaged into adenoviral capsids which are spacious enough to accommodate extra proteins. Based on protein/DNA ratio analysis performed previously in purified particles that only one 5.5kb ΔAd.AAV1 genome is packaged per capsid. This is confirmed by electron-microscopy of ΔAd.AAV1 particles, which reveals only spotted electron-dense staining associated with viral cores and extended free luminal space (see Figure 2).

293 cells are transfected with plasmids expressing Rep 68/78 under the CMV promoter and the kinetics of rep expression is determined by Western Blot with cell lysates collected at different time points after transfection. Next, these 293 cells are infected with Ad.AAV (MOI 1, 10, 100 pfu/cell) at specific time points after transfection of Rep plasmids depending on the Rep expression kinetics (e.g. 3, 6, 12, 24 . . . hours after transfection). It is important to time Ad.AAV infection exactly because viral DNA replication must be taking place or finished before Rep production reaches peak levels. In general, adenovirus DNA replication in 293 cells (infected with MOI 10) is maximal at 18 hours post-infection, followed by production of structural proteins, packaging of viral genomes, and breakdown of cellular membrane structures (which is concluded ~36-48h p.i.) (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia; van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin). Viruses are collected 48h after infection and banded by CsCl ultracentrifugation. Viral material from purified bands corresponding to

ΔAd.AAV is lysed, DNAse-treated (to liberate DNA associate Rep) and subjected to immunoprecipitation-Western Blot with Rep specific antibodies to detect co-packaged Rep. Based on theoretical calculations assuming that two Rep molecules bind per Ad genome, ~1-10ng Rep proteins is expected from Lysates of 10^{10} particles, which is within the range of detectability by Western Blot. Alternatively, co-packaged Rep may be detected based on its functional activity to mediate rescue and site-specific integration of the AAVITR transgene cassette. To test whether functional Rep protein is co-packaged into hybrid vector particles, CsCl purified ΔAd.AAV1 particles generated in 293 cells co-expressing Rep after Ad/AAV1 infection (ΔAd.AAV1+Rep) can be used for transduction studies. Three days after ΔAd.AAV1+Rep infection of the human cell line K562, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE. If efficient Rep-mediated site-specific integration of excised AAV cassettes is successful, then other ΔAd.AAV+Rep hybrid vectors with β-Gal and SNori as transgenes can be produced.

Integration studies with Rep vectors in erythroid cells:

The hypotheses behind the rational of a rep-expressing hybrid vector (ΔAd.AAV-rep) are: (1) transient Rep co-expression from ΔAd.AAV-rep vectors can enhance site-specific vector integration in human cells and (2) integration occurs via the AAV ITR(s) without the rep gene, which is placed outside the AAV cassette, thus eliminating rep expression upon vector integration. To test hypothesis 1, transduction frequencies of ΔAd.AAV/rep versus ΔAd.AAV vectors can be compared based on the formation of G418 resistant colonies and quantify site-specific integration events at different time points after infection of human and mouse cells by PFGE and PCR. To test hypothesis 2, the structure of integrated vector in transduced cell populations and single clones can be delineated by Southern analysis and by sequencing of vector/chromosomal DNA junctions. These studies can be performed with ΔAd.AAV-rep,ΔAd.AAV, and ΔAd.AAV+Rep (copackaged protein) in human K562 or HEL (for AAVS1 integration) and mouse MEL cell lines.

Cells infected with ΔAd.AAV-SNori, ΔAd.AAV-SNori+Rep or ΔAd.AAV-Snori-rep can be subjected to G418 selection. The number of G418 resistant colonies determined after 4 weeks of selection in relation to the number of initially infected cells. The selection process for colonies that did not survive continued selection due to potential rep-mediated cytotoxcicity or episomal vector expression can be monitored. If rep expression from ΔAd.AAV-SNori rep does not affect cell viability and proliferation, then more G418 resistant colonies should appear in ΔAd.AAV-SNori-rep and ΔAd.AAV-Snori+Rep. The structure of integrated vector can be determined by Southern Blot and sequencing of integration junctions.

To uncover a potential selection bias against rep producing cells after transduction with ΔAd.AAV/rep, site-specific and random vector integration events can be quantitated in cellular DNA isolated from cell populations at different time points after infection (e.g. 0.5, 1, 3, 7, 14 days). To do this, the techniques based on PFGE-Southern can be utilized. It is expected that the signal(s) for AAVS1-specific integration in ΔAd.AAV/rep infected human cells increases during the first days after infection and then remains constant over time.

In a separate study, the integration status of vector DNA (analyzed by PFGE or PCR) and the number of integrated copies (analyzed by Southern Blot) with the expression level of β-galactosidase in single clones transduced with β-Gal hybrid vectors (ΔAd.AAV-BG, ΔAd.AAV-BG+Rep, or ΔAd.AAV-BG-rep) can be correlated. Together with data obtained in the studies described in the Specification, this allows assessment of whether transcriptional silencing is associated with site-specific vector integration into the AAVS1 site.

It is not clear a priori whether the specific Rep function for vector rescue, concatemerization, and integration can efficiently occur in non-S-phase or non-dividing cells. To test whether $\Delta Ad.AAV^{fx}$, ΔAd.AAV or ΔAd.AAV-rep/+Rep vectors can integrate into non-dividing cells, transduction studies in cell cycle arrested cell cultures can be performed as described earlier.

DISCUSSION

The establishment of stable cell lines expressing Rep 68/78 at detectable levels is not possible, which is probably due to rep mediated cytotoxicity. Therefore, it is not possible to perform long-term transduction studies (e.g. G418 selection or studies in single clones) in combination with ectopic rep expression. Moreover, due to the inhibitory effect of rep on adenovirus replication, it is currently not possible to generate adenoviral vectors expressing rep under the RSV or PGK promoter.

Taken together, this indicates that co-expressed Rep may stimulate site-specific transgene integration.

__E. A Detailed Study Of Transduction/Integration Of Hybrid Vectors In Erythroid Cell Lines:__

In order to improve transduction and integration frequencies of the hybrid vectors into erythroid cell lines, a detailed study comparing various hybrid vectors have to be carried out as described below. The transduction studies are performed in K562 cells which is considered to be an adequate model to study gene transfer vehicles into erythroid cells (Floch, V., et al., 1997, *Blood Cells, Mol. and Diseases.*23, 69-87). The optimal vectors should be able to integrate into the cellular genome with a high frequency, determined by Pulse field gel electrophoresis (PFGE) and Southern blot as described in Example 4. In addition, the results from the following studies will serve to evaluate whether a given hybrid vector needs to be modified for site-specific integration in the host genome.

__Sequencing of integration junctions:__

The ultimate proof for vector integration is the sequencing of junctions between SNori vector DNA and chromosomal DNA. Furthermore, this clarifies the question whether the AAV ITRs represent the substrate for integration. Specifically, DNA from clones with

known ΔAd.AAVSNori integration structure (analyzed by Southern Blot) digested with EcoRI, which does not cut within the SNori cassette. The resulting fragments are circularized and transformed into a specific E. coli strain (according to the protocol described by Rutledge and Russell (Rutledge, E. A. et al., 1997, *Journal of Virology,* 71:8429-36)). Kanamycin resistant bacterial clones should contain the integrated SNori cassette. Flanking chromosomal DNA in rescued plasmids can be sequenced with primers specific to the transgene.

To confirm vector integration in a small number of transduced cells, genomic DNA is extracted and digested with EcoRI. EcoRI fragments are ligated to linkers containing a specific primer binding site and are then digested with NotI, religated and propagated in *E. coli*. Plasmid DNA from a representative number of bacterial clones is sequenced to determine the vector/chromosomal DNA junctions.

Dose dependent toxicity:

In order to test that the transduction frequence is dose-dependent and ΔAd.AAV vectors, which are devoid of all adenoviral genes, could be used to infect cells at higher doses with less cytotoxicity than first generation adenovirus, K562 cells are infected with different MOIs ($1\text{-}10^8$) of ΔAd.AAVBG and the first generation vector Ad.AAVBG (which contains the same β-Gal expression cassette). At day 4 post-infection, the total number of cells, the percentage of viable cells (based on trypan blue exclusion) and the percentage of X-Gal positive cells are counted. A fraction of infected cells are quantified for β-Gal expression using the Galacto-Light kit. The level of transgene expression is expected to be comparable between the two vectors. K562 cells are predicted to tolerate higher doses of ΔAd.AAVBG better than Ad.AAVBG which express viral genes.

Integration frequency with and without G418 selection:

In order to investigate the integration frequency of the different vectors and to confirm that AAV ITRs present in double-stranded adenoviral DNA genomes can mediate vector

integration with a frequency comparable to rAAV vectors. integration studies are performed based on the formation of G418 resistant colonies with ΔAd.AAVSNori, AdSNori, Ad.SNoriITR, and rAAVSNori after infection with $2x10^5$ and $2x10^6$ genomes per cell (Fig. 8). After infection, cells are plated in 96 well plates under limiting dilution and selected with G418 to estimate the frequency of formation of G418 resistant colonies. Another set of cells is plated without G418. A representative number of clones (w/ and w/o G418 selection) are expanded to $>10^6$ cells (after 3-4 weeks of culture) and analyzed for the presence of viral DNA by Southern Blot as well as PFGE analysis to discriminate between episomal vector DNA and vector genomes stably associated with chromosomal DNA. This allows us to estimate the integration frequency of the different vectors, to assess the effect of G418 selection on integration, and to consider position effects on neo expression in calculating the total integration frequency. Integrated vector copies with a frequency of at least $1x10^{-4}$ is predicted only for ΔAd.AAVSNori and rAAVSNori. The total number of colonies may be lower in both the first generation vectors, Ad.AAVSNoriITR and Ad.SNori, due to the toxic effects of expressed adenoviral proteins; however, a higher integration frequency is predicted for the vector containing the AAV ITRs (Ad.AAVSNoriITR).

Kinetics of integration:

Compared to rAAV, the double-stranded nature of entering ΔAd.AAV genomes provides more protection against degradation. Furthermore, the synthesis of transcriptionally active double-stranded intermediates from single-stranded genomes, which is considered a limiting step in rAAV transduction, is not required in ΔAd.AAV transduction. Thus, the lag phase between infection and expression seen with rAAV vectors, which is causally linked to double-strand synthesis/integration may be shorter or absent in infections with ΔAd.AAV vectors. Furthermore, it was demonstrated earlier that a 9kb mini-adenoviral genome packaged into adenoviral particles is only short lived and completely degraded by day 3 post-infusion. In contrast, transduction with the 5.5kb ΔAd.AAV1 (Figure 1) genome allows for long-term expression, suggesting that either

AAV ITRs can stabilize the viral genome as an episome until it is integrated or integration occurs shortly after infection.

The status of vector DNA can be examined in K562 cells at different time points after infection with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, or rAAVSNori (MOI $2x10^5$). Infected cells are harvested at 1 hour, 5 hours, 1 day, 3, 7, and 14 days after infection and chromosomal DNA is analyzed by PFGE followed by hybridization with a transgene specific probe. This technique allows us to distinguish between episomal vector DNA, which appears as a distinct 5.0kb band and integrated DNA. Furthermore, extra chromosomal high-molecular weight vector concatemers can be detected. In the case of random integration, after digestion of chromosomal DNA with I-CeuI or PI-SceI, vector-specific signals in the range of 1-2mb should be seen. The intensity of episomal and integrated vector signal is quantified for each time point using phosphoimager analysis. This gives information about the kinetics of hybrid vector integration in a population of infected K562 cells and the intracellular stability of hybrid vector genomes.

Structure of integrated vector DNA and integration junctions with chromosomal DNA:

ΔAd.AAV1 integrates as concatemer/s randomly into host DNA as shown previously. How many vector copies are present in one concatemer and whether the extent and the kinetics of tandem-formation are dose dependent still remain unclear. Another unanswered question is how ΔAd.AAV integrates: whether one or both ITRs are involved, whether the integrated ITRs are still intact, and whether adenoviral sequences integrate as well. These issues are important for the strategy to include rep genes into the hybrid vector genome. Moreover, if intact AAV ITRs are present within integrated vector copies, helper virus (adenovirus or HSV) infection in vivomay mobilize the integrated AAV-ITR vector cassette and affect stability of transgene expression.

To answer these questions, K562 cells can be infected with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, and rAAVSNori at MOIs $2x10^5$, $2x10^6$, or $2x10^7$ genomes per cell. Infected cells are plated in 96 well plates in the presence or the absence of G418. The

latter is included because G418 may cause amplification of integrated vector DNA (Rutledge, E. A. et al., 1997, *Journal of Virology*, 71:8429-36). Genomic DNA from isolated clones can be analyzed by regular Southern Blot as described in the Examples Section to confirm the presence of vector concatemers and calculate the number of integrated vector copies. More informative is the sequencing of integrated vector copies and their junctions with chromosomal DNA. The structure of integration junctions can be delineated using the role of AAV ITRs in vector integration and the extent of insertional mutagenesis after transduction. This data provides information about the potential risks of hybrid vector used in clinical trials.

Transduction of cell cycle arrested cells:

The ultimate target for the hybrid vectors described in the Specification are quiescent hematopoietic stem cells. We hypothesize that the double-stranded nature of ΔAd.AAV genomes and specific nuclear import mechanisms may allow for the transduction of non-dividing cells. This is in part supported by the transduction studies with ΔAd.AAV1 in quiescent hepatocytes in vivo. To confirm this data, primary fibroblasts can be forced to enter the G_0 phase by serum/growth factor starvation before infection with the hybrid vectors according to a protocol described by Russell (Russell, D. et al., 1995, *PNAS*, 92:5719-23). Cells are maintained for three days after infection under serum/growth factor deprivation. At this time point, genomic DNA is isolated and analyzed for integration events by PFGE in comparison with growing cells. Another series of integration studies can be carried out on K562 cells arrested in the G_1/S phase of the cell cycle with aphidicolin (added 1 day before and maintained several days after infection with hybrid vectors - depending on the integration kinetics studies described earlier). To investigate whether DNA damaging agents increase the transduction frequency of hybrid vectors, cell-cycle-arrested K562 cells or primary fibroblasts can be treated with cisplatinum or ^{3}H-thymidine prior to virus infection according to a protocol described by Alexander and Russell (Alexander, I. E. et al., 1994, *J. Virol.*, 68:8282-87; Russell, D. et al., 1995, *PNAS*, 92:5719-23). Furthermore, the effect of chromosomal DNA decondensation on the transduction efficiency of hybrid vectors can be studied in arrested

cells after treatment with puromycin, staurosporin, Hoechst 3328, distramycin, or vandate.

F. Improvements in ΔAd.AAV Production and Purification

To inhibit packaging of full-length genomes a modified form of I-Sce I, a yeast mitochondrial intron-endonuclease with a non-palindromic 18-bp recognition sequence is expressed in 293 cells. Constitutive expression of this enzyme in mammalian cells is not toxic, possibly due to either the lack of I-SceI sites in the genome or sufficient repair of them (Rouet P. et al, 1994, *PNAS*, 91:6064-8). The yeast I-Sce I is modified with an SV40 T-antigen nuclear localization signal and an optimal Kozak sequence to enhance its functionality in mammalian cells (Rouet P. et al, 1994, *PNAS*, 91:6064-8). For another yeast endonuclease it was shown that a recognition site within an transduced Ad genome was efficiently (30% of all transduced genomes) when expressed in human A549 cells. Importantly, the expression of E4 ORF6 and ORF3 expressed from the transduced Ad genome inhibited double-strand break repair mediated by the endonuclease (Nicolas, A. L. et al, 2000, *Virology*, 266:211-24). This is consistent with the observations by others where these E4 proteins prevent concatemerization of the viral genome (Boyer, J. et al, 1999, *Virology*, 263:307-12). Based on this, packaging of full-length virus containing a I-SceI recognition site is reduced in 293 cells constitutively expressing I-Sce I. The 18mer I-Sce site is inserted into the E3 region of the Ad.IR vectors. These vectors are generated and amplified in 293 cells followed by a large-scale infection of 293 cells expressing I-SceI. Alternatively, an expression cassette for the endonuclease XhoI is inserted into the E3 region of Ad.IR or Ad.AAV vectors. The XhoI gene will be modified for optimal function in mammalian cells. Vectors expressing XhoI are generated and amplified in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Nelson, J. E. et al, 1997, *J. Virol.*, 71:8902-7), which mediates the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG. This protects the viral and cellular genome from XhoI cleavage. Methylated Ad vectors are produced at high titers. ΔAd.AAV vectors are then obtained by large-scale infection of 293 cells with the Ad.AAV-XhoI vectors. At this stage the viral

genome is not methylated and is digested at the XhoI sites. XhoI sites present within the transgene cassette are deleted by site-directed mutagenesis without altering the amino acids sequence. (XhoI is accumulated only at late stages in virus replication and should act only upon a large part of Ad DNA when replication is completed. In addition, ultracentrifugation optimizes the separation between ΔAd.IR and ΔAd.IR particles (Blague, C. et al., 2000, *Blood*, 95:820-8).

EXAMPLE II

MODIFIED FIBER PROTEIN

A. Test the Infectivity of Different Human or Animal Serotype on Human Bone Marrow Cells.

Since the amino acid sequence of the fiber knob region varies considerably among the ~50 known serotypes, it is thought that different adenovirus serotypes bind to different cellular receptor proteins or use different entry mechanisms (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14; Defer, M., et al., 1993, *J. of Virology*, 64, 3661-3673). Although most adenoviruses contain RGD motifs in the penton base proteins, there are a number of serotypes (e.g. Ad 40, 41) without this conserved sequence. These types may use integrin □v-independent pathways for virus internalization (Davison, A.J., et al., 1993, *J. Mol. Biol.* , 234, 1308-1316; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14). To test whether other Ad serotypes can infect stem cell subpopulation present in human bone marrow, studies with a series of different human Ad serotypes and animal viruses can be performed (see Table II). As a means to verify efficient transduction with Ad serotypes, viral DNA is tagged before infection and the presence of viral genomes in the nuclei of transduced cells is investigated. Furthermore, whether viral DNA is replicated in transduced cells can be analyzed as indirect proof for early viral gene expression. A direct detection of expressed viral proteins is impossible due to the unavailability of antibodies against all the serotypes included in this study. Simultaneously with the infection assay, transduced

human bone marrow cells can be analyzed for morphological and immunohistochemical features characteristic of HSC or progenitor subpopulations. For retargeting, serotypes which are able to infect CD34+ subsets of bone marrow cells at the lowest MOI are selected. As the next step, the fiber gene is PCR-cloned from serotypes with potential HSC/CD34+ tropism and inserted into standard shuttle plasmids for Ad5 vector generation replacing the Ad5 fiber gene using an E. coli recombination system (Figure 9).

METHODS

<u>Cells and viruses:</u>

HeLa (human cervix carcinoma, ATCC CCL-2.2), CHO (chinese hamster ovary, ATCC CCL-61), K562 (human hematopoietic, ATCC 45506), HEp-2 (human larynx carcinoma, ATCC CCL-23), 293 (human embryonic kidney, Microbix, Toronto Canada) cells were maintained in DMEM, 10% FCS, 2 mM glutamine, and Pen/Strep. Culture media for CHO cells was supplemented with 200μM asparagine and 200μM proline. Human CD34+-enriched bone marrow cells were purified from peripheral blood after mobilization using MiniMACS VS^+ separation columns (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. Aliquots were stored in liquid nitrogen. Sixteen hours before the experiment, cells were recovered from the frozen stock and incubated overnight in IMDM media, supplemented with 20% FCS, 10^{-4} M β-mercaptoethanol, 100 μg/ml DNaseI, 2 mM glutamine, 10 U/ml IL-3, and 50 ng/ml stem cell factor (SCF) or 2 ng/ml thrombopoietin (Tpo). The purity of CD34+ preparations was verified by flow cytometry and was consistently greater than 90%.

<u>Flow cytometry:</u>

Adherent cells (CHO, HeLa) grown in non-tissue culture treated 10 cm dishes (Falcon, Franklin Lakes, NJ) were detached by treatment with 1mM EDTA and washed three times with wash buffer (WB), consisting of PBS supplemented with 1% FCS. Cells

grown in suspension (K562, CD34+) were washed three times with WB. After washing, cells were resuspended in WB at 2 x 10^6 cells/ml. 2 x 10^5 cells were incubated in WB for 1 h at 37°C with monoclonal antibodies specific for α_v-integrins [L230, ATCC: HB-8448, (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795) (1/30 final dilution), CAR [RmcB (Bergelson, J. M., et al. 1997. *Science*. 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology*. 62:1647-1652) (1/400 final dilution)], or BrdU [(Amersham, Arlington Heights, IL) (1/100 final dilution)]. Subsequently, cells were washed with WB, and incubated with fluorescein isothiocyanate (FITC)-labeled horse anti-mouse IgG antibodies [(Vector Labs., Burlingame, CA) (1/100 final dilution)] or phycoerythrin (PE)-labeled goat anti-mouse IgG antibodies [(Calbiochem, La Jolla, CA) 1:100 dilution] for 30 min at 4°C. After incubation with secondary antibodies, cells were washed two times with WB and 10^4 cells per sample were analyzed in duplicate by flow cytometry.

For the analysis of CD34 and c-kit expression on transduced CD34+-cells and for fluorescent activated cell sorting (FACS), purified human CD34+ cells were incubated with phycoerythrin(PE)-conjugated anti-CD34 monoclonal antibodies (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA) or with PE-labeled anti-CD117 (c-kit) monoclonal antibodies (MAb 95C3, Immunotech, Beckman Coulter, Marseille, France) according to the manufacturer's protocol followed by flow cytometry analysis. All analyses and sortings were performed on a FACStar Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with 488nm argon and 633 nm HeNe lasers. For analysis of c-kit expression and FACS purification of CD34+/c-kit+ cells, SCF was not added to the media during culturing of CD34+ cells.

RESULTS

CAR/α_v-integrin expression on test cells:

It is generally accepted that CD34+ cells possess bone marrow repopulating activity. Therefore, we used human CD34+ cells as the target for our studies towards identifying Ad serotypes with HSC tropism and constructing new viral vectors. Studies were

performed on mobilized, CD34-positive, peripheral blood cells from one donor under conditions which are known to retain CD34+ cells in a quiescent stage (Leitner, A., et al. 1996. *Br.J.Haematol.* 92:255-262; Roberts, A. W., Metcalf, D. 1995. *Blood.* 86:1600-1605). More than 90% of purified cells were CD34 positive by flow cytometry. Furthermore, we included into our Ad tropism studies the cell line K562, which is considered to be an adequate model for studying gene transfer into human hematopoietic cells (McGuckin, et al. 1996. *British Journal of Haematology.* 95:457-460). HeLa cells, which are readily infectible by Ad5, and CHO cells, which are refractory to Ad5 infection (Antoniou, M. et al., 1998, *Nucleic Acid Res.,* 26:721-9), were used as positive and negative control cell lines, respectively.

For Ad5, both, binding to the primary receptor and to $\alpha_3\beta_5$ and $\alpha_{\varpi}\beta_5$ integrins are important for high efficiency infection of target cells. The expression of CAR and α_v integrins on test cells was analyzed by flow cytometry using monoclonal antibodies against CAR (RmcB (Bergelson, J. M., et al. 1997. *Science.* 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology.* 62:1647-1652)) and α_v integrins (L230 (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621)) (Figure 10). As expected, nearly all HeLa cells expressed high levels of CAR and α_v-integrins, whereas CHO cells lacked significant CAR and α_v-integrin expression. Fifteen and 77% of K562 cells expressed CAR and α_v-integrins, respectively. Only ~6% of the CD34+ cells used in our studies expressed CAR and 17% were positive for α_v-integrins. Notably, the preparation of CD34+ cells represents a mixture of different cell types. The absent or low expression of primary and secondary Ad5 receptors on non-cycling human CD34+ cells is in agreement with previous reports (Huang, S., et al. 1996. *J. Virology.* 70:4502-4508; Neering, S. J., et al. 1996.. *Blood.* 88:1147-1155; Tomko, R. P., et al.. 1997. *Proc. Natl. Acad. Sci. USA.* 94:3352-3356).

Infection assay using wild-type Ad5 and K562 cells:

The presence of viral DNA in the nucleus of infected cells is an indirect means to demonstrate efficient virus binding, internalization, and nuclear import. Nuclear

localization of the viral genome is a prerequisite for transgene transcription and integration. Two techniques are utilized to tag viral DNA for in situ analysis. To optimize the infection assay, wild-type Ad5 virus and K562 cells which are permissive for Ad5 infection can be used. The first protocol (Challberg, S.S. and Ketner, S. 1981, *Virology* 114, 196-209), is based on ^{32}P-labeling of viral DNA. During amplification of wild-type Ad5 and A549 cells, ^{32}P-phosphate (40μCi/ml) is added to phosphate-free medium. After development of CPE, ^{32}P-tagged virus is harvested, banded in CsCl gradients, and titered on HeLa cells according to standard protocols. To simulate the conditions for infection of human bone marrow cells, K562 cells are incubated in suspension with a MOI of 1, 10, or 100 of ^{32}P-Ad5 for 2, 4, 6, or 8 hours under agitation at 37°C. This covers the time period necessary for adsorption, internalization, and nuclear import. After washing, cells are fixed either transferred to microscopy slides using cytospin or embedded in paraffin and sectioned (according to protocols from VECTOR labs, Burlingham, CA). The latter has the potential advantage that multiple consecutive sections (5μm) of the same cell can be analyzed by different methods (e.g. for ^{32}P tagged viral DNA, for specific histological staining, for immunofluorescence), which allows for correlating infection with a particular cell type present in the bone marrow. Cells are incubated in a Kodak NTB-2 photo emulsion for autoradiography. The exposure time can be optimized to minimize background or non-nuclear localized signals. A dose and time dependent appearance of nuclear silver grains is expected under the optimized conditions. Since ^{32}P-phosphate can label viral proteins as well, a cytoplasmic background signal might appear. To facilitate detection, immunofluorescence with HSC specific antibodies on sections can be performed. As an alternative method, a BrdU-labeling technique for viral DNA can be used (Lieber, A., et al. 1999. *J Virol* 73:9314-24; Lieber, A. et al., 1996, *Journal of Virology*, 70:8944-60). In this case, different amounts of BrdU are added to the A549 culture medium during wtAd5 virus propagation. BrdU labeled viral DNA can be detected with monoclonal antibodies specific to BrdU. The signal can be enhanced using layers of species-specific polyclonal antibodies in combination with biotin/avidin and a fluorescent marker. BrdU tagged viral DNA can be detected on cytospins of bone marrow cells together with cell surface markers by double or triple immunoflourescence.

DISCUSSION

The interaction of selected Ad serotypes with CD34+ cells was tested. As a result of this screening we constructed a first-generation, Ad5-based vector whose fiber was substituted with the fiber derived from Ad35. We demonstrated that this capsid modification allowed for efficient viral transduction of potential HSCs by the corresponding chimeric Ad vectors.

All tropism and transduction studies were performed with non-cycling CD34+ cells, which are thought to include HSCs. The quiescent stage of CD34+ cells purified from mobilized blood is important because induction of cell proliferation is associated with a loss of the ability to reconstitute hematopoiesis and with changes in the spectrum of cellular receptors (Becker, P. S., et al. 1999. *Exp. Hematol.* 27:533-541). It is known that treatment of hematopoietic cells with cytokines or growth factors changes the expression of specific integrins including α_v-integrins, which would ultimately alter the susceptibility of cells to Ad infection or may effect viability of infected cells (Gonzalez, R., et al. 1999. *Gene Therapy.* 6:314-320; Huang, S., et al. 1995. *J. Virology.* 69:2257-2263). Another fact that complicates the interpretation of transduction studies is the extraordinary heterogeneity of CD34+ cells in regards to morphology and function.

B. Screening different adenoviruses to establish tropism to HSC.

The ATCC provides more than 70 different human or animal adenoviruses (see Appendix I). A collection of 15 human serotypes and 6 animal adenoviruses (see Table II) are selected based on the following criteria: (i) availability of the complete genome sequence or fiber sequence from the NIH gene bank (ii) CAR receptor usage absent or unknown, (iii) different subgroups, and (iv) moderate or low tumorigenicity (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia). However, any serotype shown in the Appendix hereto can be used for the invention described. Animal viruses are included in the infectivity assay because this may provide a

means to circumvent the pre-existing humoral immunity against human Ad5 fiber, which represents a critical obstacle for clinical trials with Ad vectors.

METHODS

Viruses:

The following human adenovirus serotypes were purchased from the ATCC: 3 (VR-3), 4 (VR1081), 5 (VR-5), 9 (VR1086), 35 (VR-716) and 41(VR-930). Adenovirus No. VR-716 was purchased from ATCC labeled as serotype 34, however it was found to be serotype 35 upon sequencing of the fiber region. For amplification, the corresponding Ads were infected onto HeLa, 293, or HEp-2 cells under conditions that prevented cross-contamination. Virus was banded in CsCl gradients, dialyzed and stored in aliquots as described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaque titering was performed as follows: Confluent 293 cells plated in 6-well plates were incubated for 24 hours with virus in a total volume of 1ml. Two weeks after infection, plaques were counted on cultures overlayed with 1% agarose/MEM/10% FCS.0

EM studies:

CsCl-banded Ad stocks were thawed and diluted with 0.5% glutaraldehyde. Grids were prepared as described earlier (Mittereder, N., et al. 1996. *J. Virology*. 70:7498-7509). After staining with 2% methylamine tungstate (Nanoprobes, Stony Brook, NY), the carbon-coated grids were evaluated and photomicrographed with a Phillips 410 electron microscope, operated at 80 kV (final magnification 85,000x). For each particular Ad serotype, the number of morphologically deficient viral particles per 100 was counted in five random fields.

RESULTS

Electron microscopy:

Little is known about the stability of particles from serotypes other than Ad5. Since the intactness of viral particles was crucial for comparative interaction studies, virions from the serotypes specified above were analyzed by electron microscopy (EM). EM studies of negative contrast stained Ad suspensions demonstrated that the percentage of defective particles (loss of icosahedral shape or luminal staining) did not exceed 5% indicating that serotype preparations had comparable qualities. Representative EM photographs are shown for Ads 5, 9, and 35 (Figure 11).

Serotype screening:

It is thought that different Ad serotypes bind to different cellular receptor proteins and use different entry mechanisms (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Mathias, P., et al. 1994. *Journal of Virology*. 68:6811-6814). A set of human adenoviruses was obtained from the ATCC to be tested for tropism to CD34+ cells. These included serotypes 3, 4, 5, 9, 35, and 41 representing different subtypes (Table 1). We believed that these serotypes would use different cellular attachment and internalization strategies due to differing lengths of fiber shafts (Chroboczek, J., et al. 1995. *Adenovirus fiber*, p. 163-200. *In* a. P. B. W. Doerfler (ed.), *The molecular repertoire of adenoviruses*, vol. 1. Springer Verlag, Berlin; Roelvink, P. W., et al. 1998. *J. Virology*. 72:7909-7915), the presence or absence of RGD motifs within the penton base, and differing tissue tropism. The relatively little characterized Ad35 was selected because it was found in immunocompromised hosts, particularly in bone marrow recipients (Flomenberg, P., et al. 1994. *Journal of Infectious Diseases*. 169:775-781; Flomenberg, P. R., et al. 1987. *Journal of Infectious Diseases*. 155:1127-1134; Shields, A. F., et al. 1985 New England *Journal of Medicine*. 312:529-533). The latter observations prompted us to believe that bone marrow cells are among the natural reservoirs for Ad35.

TABLE II

Human and animal adenoviruses with potential interest for the invention

Adeno virus	Human/ Group B	Human/ Group D	Human/ Group F	Avian	Bovine	Canine	Ovine	Swine	Mouse
Sero type	3,7,11,16, 21,34,35	8,15,17, 19,28,37	40, 41	CELO, EDS	3	1,2	5	4	1

The underlined serotypes use CAR independent pathways for cell entry.

For amplification, the corresponding adenovirus stocks can be infected onto HeLa or A549 cells such that at a given time only one virus type is handled in a separate laminar flow hood and cultured in Hepa-filtered bottles, preferentially in separate CO_2 incubators to avoid cross-contamination. During propagation, viral DNA is tagged using one of the techniques described earlier. Viral DNA can be isolated from purified particles. The XhoI restriction pattern is analyzed for methylated and unmethylated viral DNA by Southern blot using the full genome of the corresponding virus type as a radioactive probe.

DISCUSSION

Although it was reported earlier by slot-blot assay that fiber knobs derived from 2, 9, 4, and 41L can bind to CAR (Roelvink, P. W., et al.. 1998. *J. Virology*. 72:7909-7915), it is not clear whether this binding occurs with an affinity that is physiologically relevant and whether this would confer cell entry. Furthermore, as shown for the Ad5 interaction between the penton and intergrins, a secondary receptor is required to induce virus internalization. We demonstrated that different serotypes interacted differently with the K562 or CD34+ target cells. Ad5, Ad4, and Ad41 were not able to efficiently attach to and be internalized by K562 and CD34+ cells. Although Ad4 belongs to a separate subgroup (E), it is thought that Ad4 represents a natural hybrid between subgroup B and C viruses with a fiber related to Ad5 (Gruber, W. C., et al. 1993. *Virology*. 196:603-611).

Therefore, it was not surprising that Ad4 has binding properties similar to Ad5. The subgroup F serotype Ad41 has been shown to contain distinct fibers, a long shafted and a short-shafted fiber allowing for different cell entry pathways (Tiemessen, C. T., Kidd, A.H. 1995. *J. Gen. Virol.* 76:481-497). The Ad41 penton base does not contain RGD motifs suggesting that this virus may use α_v-intregrin independent pathways for cell entry. However, these features did not improve interaction with CD34+ cells. Ad9, Ad3, and Ad35 did interact with CD34+ cells more efficiently than Ad5. Out of all the serotypes tested, Ad35 demonstrated the most efficient attachment and internalization with K562 and CD34+ cells. Although the short-shafted Ad9 can bind to CAR, it preferentially uses α_v-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Therefore, the low level of α_v-integrin expression on certain subset/s of CD34+ cells may account for the observed susceptibility to Ad9.

<u>**C. Attachment and Internalization of the Ad serotypes to K562 and CD34+ cells.**</u>

METHODS

<u>*Labeling of Ads with [^{3}H]-methyl thymidine:*</u>

Serotypes were labeled with [^{3}H]-methyl thymidine as described in detail elsewhere (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Briefly, 5×10^7 HeLa or 293 cells were grown in 175 sq. cm flasks with 15 ml DMEM/10% FCS and infected with wild type adenovirus at a MOI of 50 or higher. Twelve hours post-infection, 1 mCi of [^{3}H]-methyl thymidine (Amersham, Arlington Heights, IL) was added to the media and cells were further incubated at 37°C until complete CPE was observed. Then, cells were harvested, pelleted, washed once with cold PBS, and resuspended in 5 ml PBS. Virus was released from the cells by four freeze-thaw cycles. Cell debris was removed by centrifugation and viral material was subjected to ultracentrifugation in CsCl gradients and subsequent dialysis as previously described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology.* 70:8944-8960). Virus purification and dialysis removed unincorporated radioactivity. Wild type Ad particle concentrations were determined

spectrophotometrically by measuring the OD_{260}, utilizing the extinction coefficient for wild-type Ad5 $\varepsilon_{260} = 9.09 \times 10^{-13}$ OD ml cm virion^{-1} (Maizel, J. V., et al. 1968. *Virology*. 36:115-125). The virion specific radioactivity was measured by a liquid scintillation counter and was always in the range of 1×10^{-5} to 1×10^{-4} cpm per virion. For selected variants, the fiber gene was PCR amplified and sequenced to ensure identity and the absence of cross-contamination.

Viral DNA tagged with methylase and test for replication by genomic Southern blots:

To ultimately confirm transduction, a protocol to detect adenoviral replication in infected cells can be established. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. A site-specific methylation strategy is utilized to monitor viral DNA replication within infected cells (Nelson, J. et al., 1997, *Journal of Virology*, 71:8902-07). Methylation marked adenovirus can be produced by the addition of a methyl group onto the N6 position of the adenine base of XhoI sites, CTCGAG, by propagation of the virus in HeLa or A549 cells expressing the XhoI isoschizomer PaeR7 methyltransferase (PMT) (Kwoh, T.J., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 7713-7717). It is known that methylation does not affect vector production but does prevent cleavage by XhoI. Loss of methylation through viral replication restores XhoI cleavage and can be detected by Southern blots of genomic DNA from infected cells in comparison to native, non-methylated, viral genomes.

Attachment and internalization assays:

These studies were performed based on a protocol published elsewhere (Wickham, T. J., et al. 1993. *Cell*. 73:309-319). In preliminary experiments, we found that labeled Ad5 virions reached equilibrium in attachment to HeLa cells after 45 min at 4°C with an MOI of 400 pfu per cell. For attachment studies, 3.5×10^5 cells were incubated for one hour on ice with equal amounts of [^{3}H]-labeled adenovirus OD particles equivalent to an MOI of 400 pfu/cell for Ad5 in 100 µl of ice-cold adhesion buffer (Dulbeco's modified Eagle's medium supplemented with 2 mM $MgCl_2$, 1% BSA, and 20 mM HEPES). Next, the cells

were pelleted by centrifugation for 4 min at 1000 x g and washed two times with 0.5 ml ice-cold PBS. After the last wash, the cells were pelleted at 1500 x g, the supernatant was removed, and the cell-associated radioactivity was determined by a scintillation counter. The number of viral particles bound per cell was calculated using the virion specific radioactivity and the number of cells. To determine the fraction of internalized [^{3}H]-labeled adenoviral particles, cells were incubated on ice for one hour with the corresponding virus, washed with PBS as described above, resuspended in 100 μl adhesion buffer, and then incubated at 37°C for 30 min. Following this incubation, cells were diluted 3-fold with cold 0.05% trypsin-0.5mM EDTA solution and incubated at 37°C for an additional 5-10 min. This treatment removed 99% of attached radioactivity. Finally, the cells were pelleted at 1500 x g for 5 min, the supernatant was removed, and the protease-resistant counts per minute were measured. This protocol minimizes the possibility that the internalization data were affected by receptor recycling (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795). Nonspecific binding of Ad particles to cells on ice was determined in the presence of 100-fold excess of unlabeled virus. This value routinely represented less than 0.1 % of viral load.

RESULTS

Attachment of Ad particles to target cells and internalization:

The selected serotypes were metabolically labeled with [^{3}H]-thymidine, which is incorporated into viral DNA during replication. Adsorption and internalization can be experimentally dissociated by taking advantage of the observation that at low temperature (0-4°C) only virus cell attachment occurs, whereas internalization requires incubation at higher temperatures. The number of particles adsorbed or internalized per cell was calculated using the virion-specific radioactivity and used to quantify interaction of Ads 3, 4, 5, 9, 35, and 41 with CD34+, K562, HeLa and CHO cells (Figure 12). The serotypes varied significantly in their ability to attach to and to be internalized by the different cell lines. For Ad5, the degree of attachment to the cell lines tested correlated with the level of CAR expression. In CHO cells, which were previously shown to be refractory to Ad5

infection, the level of attachment and internalization was about 50-70 viral particles per cell. This number was hereafter assumed negative in terms of susceptibility of a given cell type for Ad5. Interaction of the other serotypes with CHO cells was not significantly higher indicating that corresponding receptor/s were absent on CHO cells. All serotypes tested interacted with HeLa cells; with Ad3 and Ad35 being the most efficient variants. The presence of distinct Ad3 and Ad5 receptors on HeLa cells was demonstrated previously (Stevenson, S. C., et al. 1995. *J. Virology*. 69:2850-2857). Ads 4, 5, and 41 did not bind to K562 cells. In contrast, Ad9 as well as the members of subgroup B, Ad3 and Ad35, efficiently interacted with K562 cells with Ad35 having the highest number of adsorbed and internalized particles. Compared to Ad5, about 25 times more Ad35 particles were attached and three-forth of these were internalized by K562 cells. Viral interactions with CD34+ cells were generally weaker. Among the serotypes tested, only Ad9 and Ad35 were significantly internalized by non-cycling CD34+ cells. Internalization of Ad9 and Ad35 was, respectively, four and eight times more efficient than for Ad5 particles. The number of Ad35 virions internalized by CD34+ cells was almost half of that seen for Ad5 in HeLa cells, which can be readily infected with Ad5 based vectors.

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into HeLa, 293, and CHO cells:

Hela and 293 cells expressing high level of primary and secondary receptors for human adenoviruses are used as a positive control for virus attachment and internalization. As a negative control CHO cells are used. CHO cells do not express the primary adenoviral receptor at a detectable level, and are therefore refractory for adenoviral infection. For attachment studies, these adherent cell lines are detached from 10 cm dishes with PBS-EDTA solution (without Ca2+ and Mg2+), washed three times with ice-cold PBS, resuspended in adhesion buffer, and incubated with viruses as described above in the Examples section. As expected, all adenoviral serotypes tested are efficiently attached to and internalized into Hela cells (Table III) (Figure 13). Adenoviruses serotypes 3, 5, 35, 41, but not 9, are efficiently attached to and internalized by 293 cells. In contrast, poor

attachment and internalization of most adenovirus serotypes are observed with CHO cells. The level of attachment on CHO is about 50-70 virus particles per cell for adenoviruses serotypes 5 and 41, 115 virus particles per cell for adenovirus type 3 and about 180 particles per cell for adenovirus serotypes 9 and 35. For further analysis, numbers >300 viral particles per cell are assumed as positive and <70 viral particles per cell as negative in terms of susceptibility of a particular cell line for efficient adenoviral transduction.

TABLE III

Comparative analysis of attachment and internalization of Ad5 and Ad9 to cell lines, expressing different amounts of CAR and αυβintegrins.

Cell line	CAR expression	αυβ-integrin expression	Ad9 (attached/ internalized)	Ad5 (attached/ internalized)
HeLa	++	++	426/370	550/500
CHO	-	++	300/300	70/50
293	++	++	20/20	1950/1750
Y79	+++	-	190/140	1200/1100
K562	-	+	320/230	60/50
Erythrocytes	?	?	420/-	68/-

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into human CD34+ bone marrow cells and K562 erythroleukemia cell line:

Previous studies showed that the human erythroleukemia cell line K562 can be transduced with Ad5-based adenoviral vectors at very high MOIs. As shown in Figure 14, only about 60 viral particles per cell of adenovirus serotype 5 are attached to and even fewer particles are internalized into these cells at a MOI of 400. In contrast to Ad5, about 320 viral particles per cell of Ad9 and about 1500 viral particles per cell of Ad35 are

attached to and about two-thirds of them are internalized into K562 cells (Figure 14B). Human unstimulated CD34+-enriched bone marrow cells obtained from frozen stocks are incubated overnight in growth medium without cytokine stimulation. The next day, the number of viable cells is calculated. For attachment studies, cells are washed three times with ice-cold PBS, resuspended in adhesion buffer and incubated with adenoviruses. Among the adenoviral serotypes tested, only adenovirus particle of Ad9 (about 150 viral particles per cell) and Ad35 (about 320 viral particles per cell) are able to attach to unstimulated CD34+ cells on the level, compared to Ad 5 (only 60 viral particles per cell). Four-fifths of these virus particles are able to be internalized by the cells. Interestingly, upon stimulation of CD34+ cells with GM-CSF and EPO/TPO for two weeks, attachment and internalization of Ad9 viral particles are significantly increased (up to 300 particles per cell). At the same time, the transient stimulation of cells with GM-CSF for two days could not increase the level of viral attachment to the cells.

Based on the above finding that Ad35 serotype is able to attach and internalize into CD34+ cells most efficiently among several serotypes tested, serotype Ad35 was selected for further studies. As described in Appendix II, a chimeric vector (Ad5 GFP/F35) containing the short-shafted Ad35 fiber sequence in an Ad5 capsid was able to target a broad spectrum of CD34+ cells in a CAR/integrin independent manner.

DISCUSSION

In summary, from all the serotypes tested, Ad9, Ad3, and Ad35 demonstrated the most efficient attachment to and internalization with K562 and CD34+ cells. Based on adsorption/internalization data, Ad9 and Ad35 as representatives for subgroups D and B were selected for further tropism studies.

D. Characterization of Ad vector replication in K562 and CD34+ cells.

Comparative analysis of Ad5, and Ad9 and Ad34 to infect and to replicate in 293, K562 and CD34+ cells. The ability of the Ad9 fiber knob domain to recognize the same

primary receptor on the cell surface as Ad5 with comparable affinity was described earlier. Thus, the finding that Ad9 viral particles can only poorly attach to 293 cells is rather unexpected. In order to find out how the attachment and internalization data reflect the biological activity of adenoviruses of different serotypes, the stocks of Ad5, Ad9 and Ad35 are characterized in more detail by electron microscopy, plaque assay on 293 cells, and quantitative replication assay in K562 and CD34+ cells.

METHODS

Quantitative replication assay:

$1x10^5$ CD34+ or K562 cells were infected in 100µl of growth media with different MOIs of Ad5, 9, or 35 which had been amplified in 293 cells, expressing the XhoI DNA methyltransferase isoshizomer PaeR7 (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). After 2 hours of incubation at 37°C, the cells were centrifuged at 1000 x g for 5 min, the virus-containing medium was removed, the cells were resuspended in 100µl of fresh media, and then they were incubated at 37°C until harvesting. At 16 hours post-infection for K562 cells, or 36 h post-infection for CD34+ cells, 5 µg of pBS (Stratagene, La Jolla, CA) plasmid DNA was added as a carrier which could also be used as a loading control. Genomic DNA was extracted as described previously (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). One-fourth of purified cellular DNA (equivalent to 2.5 x 10^4 cells) was digested with HindIII, XhoI, or with HindIII and XhoI together at 37°C overnight and subsequently separated in a 1% agarose gel followed by Southern blot with chimeric Ad5/9 or Ad5/35 DNA probes. The chimeric probes, containing sequences of Ad5 and Ad9 (Ad 5/9) or Ad5 and Ad35 (Ad 5/35), were generated by a two-step PCR amplification using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) and viral DNA from purified particles as a template. The following primers were used for PCR (Ad5 sequences and nucleotide numbers are underlined): Ad5F1 – <u>(nt: 32775-32805)</u> 5'-<u>GCC CAA GAA TAA AGA ATC GTT TGT GTT ATG</u>-3'; Ad5R1 – <u>(nt: 33651-33621)</u> 5'-<u>AGC TGG TCT AGA ATG GTG GTG GAT GGC GCC A</u>-3'; chimeric Ad5/9F – (nt: <u>31150-31177</u>, nt: 181-208) 5'-<u>AAT</u>

GGG TTT CAA GAG AGT CCC CCT GGA GTC CTG TCA CTC AAA CTA GCT GAC CCA -3'; chimeric Ad5/9R – (nt: 32805-32775, nt:1149-1113) 5'- CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC TTC ATT CTT GGG CGA TAT AGG AAA AGG-3; chimeric Ad5/35F – (nt: 31150-31177, nt: 132-159) 5'-AAT GGG TTT CAA GAG AGT CCC CCT GGA GTT CTT ACT TTA AAA TGT TTA ACC CCA-3', chimeric Ad5/35R (nt: 32805-32775, nt: 991-958) 5'-CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC ATT TTA GTT GTC GTC TTC TGT AAT GTA AG-3'. Nucleotide numbers are given according to the sequences obtained from the NCBI GenBank (accession No. M73260 / M29978 for Ad5, X74659 for Ad9, and U10272 for Ad35). After the first amplification, the 968 bp-long Ad9, a 859 bp-long Ad35 DNA fragments corresponding to the fiber genes, and a 876 bp-long Ad5 fragment corresponding to the Ad5 E4 region (located immediately downstream of Ad5 fiber gene) were purified by agarose gel electrophoresis. To generate chimeric DNA probes, amplified Ad5 DNA was mixed with Ad9 or Ad35 fragments obtained during the first step of PCR, and subjected to a second PCR amplification using Ad5/9F or Ad5/35F primers and the Ad5R1 primer. The resulting Ad5/9 or Ad5/35 chimeric DNA fragments (see Fig. 15) were purified and their concentrations were measured spectrophotometrically. Corresponding chimeric DNA fragments were loaded as concentration standards on agarose gels or labeled with [^{32}P]-dCTP and used as probes for Southern analysis. The number of viral genomes per DNA sample was calculated after quantitative Phospho-imager analysis. In preliminary experiments, no preferential hybridization of chimeric DNA probes to DNA of any particular viral serotype was detected.

RESULTS

Replication of selected serotypes in K562 and CD34+ cells:

Adsorption/internalization studies do not ultimately prove viral transduction, a process often defined as gene transfer that allows for viral or heterologous gene expression in host cells. Intracellular trafficking, including endosomal lysis, transport to the nucleus,

and nuclear import of the viral genome, depends on structural capsid proteins and thus, varies between different serotypes (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Miyazawa, et al. 1999. *J. Virology*. 73:6056-6065). We believed that analysis of viral gene expression would be a means to verify successful nuclear import of viral genomes and that this would be a good criterion for selection of serotype/s able to efficiently infect our target cells. To do this, we used a protocol, which allows for the detection of Ad replication in infected cells. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. We utilized a site-specific methylation strategy to monitor viral DNA replication within infected cells (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). Methylated Ad serotypes were produced by the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG, during propagation of the viruses in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Kwoh, T. J., et al. 1986. *Proc.Natl.Acad.Sci. USA*. 83:7713-7717) (293 PMTcells). Loss of methylation through viral replication restores Xho I cleavage and can be detected by Southern blots of Xho I-digested genomic DNA from infected cells.

Ad replication studies were performed in K562 and CD34+ cells with Ad9 and Ad35, in comparison to Ad5. For replication studies, the infectious titer (in pfu/ml) and genome titer (in genomes per ml) were determined (by plaque assay on 293 cells or by quantitative Southern blot, respectively) for methylated and unmethylated Ad5, Ad9, and Ad35 (Table 2). The ratio of pfu to genome titer was comparable for methylated and unmethylated virus demonstrating that DNA methylation had not altered transduction properties. About 85% of (Ad5, 9, and 35) virus used for infection was methylated as calculated based on the intensity of fragments specific for methylated and non-methylated viral DNA present in the viral load (Fig. 15). The numbers of genomes detected after adsorption (1 hour, 0°C) or internalization (2 hours 37°C) correlated well with studies shown in Fig. 12. Ad9 and Ad35 interacted more efficiently than Ad5 with K562 and CD34+ cells. Dose-dependent replication studies in K562 and CD34+ cells were performed with the same genome numbers of Ad5, 9, and 35 (Fig. 15). The replication rate was measured based on the ratio of methylated to demethylated viral

DNA after infection with different MOIs (2100, 420, and 105 genomes per cell). In K562 cells, efficient replication (100% conversion from methylated to unmethylated DNA) was detected for Ad5 at MOI >/= 2100, for Ad9 at MOI >/=420, and for Ad35 at MOI >/=105. This demonstrated that Ad35 transduced K562 cells with the highest efficiency. In CD34+ cells, the replication rate was 100% for Ad5 and 31% for Ad9 after infection with MOI 420. Although methylated Ad35 viral DNA was present in CD34+ cells, viral replication was undetectable for Ad35. In summary, while viral replication studies in K562 cells confirmed data obtained for Ad5, 9, and 35 adsorption and internalization, there was a discrepancy between earlier results and the poor replication of Ad9 and, particularly, Ad35 in CD34+ cells. As outlined later, replication analysis in heterogeneous cell populations, like CD34+ cells, may not allow for definitive conclusions on tropism of a particular serotype.

Taking all the screening data together, Ad9 and Ad35 emerged as the variants with the strongest tropism for K562 and CD34+ cells. It is thought that Ad9 can bind to CAR, however, it preferentially uses α_V-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology*. 70:7614-7621). This entry strategy may be not optimal for efficient infection of CD34+ cells as only less that 17% of them express α_V-integrins (Fig. 10). Therefore, we decided to concentrate on Ad35 as a source for heterologous fiber to be used for construction of a chimeric vector based on an Ad5 backbone.

TABLE IV

Results from the infectivity assay which determines the optical particle-to-PFU (OPU/PFU) ratio using 293 cells

Virus	OPU (A260)	PFU	OPU/PFU ratio
Ad5	1.4×10^{12}	1.06×10^{11}	13
Ad9	4.61×10^{11}	2.6×10^{8}	1773

DISCUSSION

Viral replication studies in K562 cells confirmed the data obtained for Ad5, 9, and 35 adsorption and internalization. However, there was a discrepancy between the interaction data and the replication data in CD34+ cells where Ad9 replicated only poorly and no replication was seen for Ad35. Ad replication is only initiated upon the production of a critical threshold of early viral proteins, which in turn, is directly dependent on the number of viral genomes present in the nuclei of infected cells. Therefore, the outcome of replication studies may be affected by the rate of nuclear import of viral genomes, by the activity of viral promoters, and/or the intracellular stability of viral DNA/RNA. These parameters may vary, on one hand, between different subsets of CD34+, and/or, on the other hand, between different Ad serotypes. In conclusion, the viral replication analyses performed with different Ad serotypes in CD34+ cells may not predict the actual transduction properties of chimeric vectors based on Ad5 backbone. This implies that attempts to produce gene transfer vectors based on Ad genomes other than Ad5 should be exercised with caution.

Recently, an Ad serotype screening strategy was used to identify variants with tropism for primary fetal rat CNS cortex cells or human umbilical vein endothelial cells. The optimal serotype (Ad17) was selected based on immunohistochemistry for hexon production 48 hours after infection (Chillon, M., et al. 1999. *J. Virology.* 73:2537-2540). However, this approach is problematic because, at least in our hands, antibodies developed against Ad5 hexon did not cross-react with other serotypes. Also, hexon is expressed only after onset of replication. As outlined above, the kinetics of intracellular trafficking, viral gene expression, and replication significantly vary between serotypes (Defer, C., et al., P. 1990. *J. Virology.* 64:3661-3673; Miyazawa, et al. 1999. *J. Virology.* 73:6056-6065).

In addition to being the most efficient serotype in terms of interaction with CD34+ cells, Ad35 is also interesting because it interacts with receptor/s different from the Ad5 and Ad3. Ad35 and Ad5GFP/F35 attachment was not inhibited by Ad5 or anti-CAR antibodies suggesting that Ad35 binding was CAR independent. First, Ad5 did not compete with Ad35 and Ad5GFP/F35 during internalization and infection indicating that

$\alpha_{v}\beta_{3/5}$- integrins are not involved in viral entry. Second, function-blocking antibodies against α_v-integrins did not compete with Ad35 and Ad5GFP/F35 for internalization into K562 cells, whereas these antibodies did inhibit Ad5 internalization. And third, in contrast to Ad5 based vectors, GFP expression after infection with Ad5GFP/F35 was not restricted to α_v–integrin-expressing CD34+ cells. From these facts, we conclude that infection with Ad35 and the chimeric Ad5GFP/F35 vector does not involve α_v-integrins. In this context, the presence or absence of RGD motifs within Ad35 penton base remains to be determined by sequencing the corresponding genome region. Cross-competition assays demonstrated that Ad35 and Ad5GFP/F35 bind to a receptor that is different from the Ad3 receptor. Although Ad3 and 35 belong to the same subgroup, they have been divided into two DNA homology clusters, B1 and B2; the amino acids composing their fibers are only 60% homologous. Furthermore, the target tissues for both viruses are different; Ad3 can cause acute respiratory infections, whereas Ad35 is associated with kidney infection (Horwitz, M. S. 1996. *Adenoviruses*, p. 2149-2171. *In* B. N. Fields, Knipe, D.M., Howley, P.M. (ed.), *Virology*, vol. 2. Lippincott-Raven Publishers Inc., Philadelphia). Therefore, it was not surprising to see that Ad3 and Ad35 recognize different receptors.

In conclusion, Ad35 and the chimeric vector enter the cells by a CAR- and α_v-integrin independent pathway. We believe that Ad35 and the chimeric vector binds primarily to its fiber receptor and that this interaction is sufficient to trigger internalization. On the other hand, Ad35 internalization may involve cellular proteins other than α_v-integrins. These membrane proteins can overlap with those for Ad3 internalization and represent β2 integrins, which protrude more from the cell surface than α_v-integrins (Huang, S., et al. 1996. *J. Virology*. 70:4502-4508).

According to EM studies of negative contrast-stained adenoviral suspensions, the percentage of deficient particles for all adenoviral serotypes tested does not exceed 5%. However, plaque assays reveal that the ability to form plaques in 293 cells is significantly different for tested serotypes. The optical particle-to-PFU (OPU/PFU) ratio obtained is 13 for Ad5, which is in good agreement with the previously estimated ratio for this

adenoviral serotype. Importantly, this ratio is about three times higher for adenovirus serotype 35 and more than 150-fold higher for adenovirus serotype 9. Furthermore, quantitative Southern blot using chimeric Ad5/9 and Ad5/35 DNA probes is used to determine the ratio between the genome and transducing titer. This study confirms the data obtained by plaque assay. Quantitative replication assay of these adenoviruses in K562 and CD34+ cells also confirms the ability of Ad9 and Ad34 to more efficiently attach to these cell types. The replication of viral genomes is observed for Ad9 and Ad34 at lower MOIs of infection, compared to Ad5. In conclusion, the data obtained for different serotypes in attachment and internalization are in good agreement with the infectivity data in target cells.

E. Attachment and internalization of different adenoviral serotypes into primary dendritic cells, JAWSII, MCF-7 and REVC cells.

As a proof of principle, the serotype screening strategy can be employed for other important target cells which are refractory to Ad5 infection.

RESULTS

RECV cells are endothelial cells which have to be targeted for approaches that are aimed to gene therapy of restenosis, atherosclerosis, inflammation etc. MCF-7 cells are breast cancer cells isolated from liver metastases which are important targets for tumor gene therapy. The human adenovirus serotypes 3, 5, 9, 35 and 41 are tested to see whether they can attach to and can be internalized by mouse primary dendritic cells, JAWSII cells, MCF-7-human breast cancer cells and REVC endothelial cells. None of the adenoviral serotype tested can efficiently attach to primary dendritic cells. Adenovirus serotype 3 is able to efficiently attach to REVC endothelial cells (about 400 virus particles per cell are attached and about 300 are internalized). In comparison, only 50 Ad5 particles are able to attach to and even fewer are internalized in these REVC. The human breast cancer cells (MCF-7) are previously shown to be refractory to Ad5

infection at low MOIs. However, Ad3 and more efficiently, Ad35 attach to and internalize into MCF-7 cells.

DISCUSSION

The data presented herein indicate that different human adenovirus serotypes recognize different cellular receptors and can therefore infect cell types that are refractory to Ad5 infection. There are adenoviral serotypes that can more efficiently attach and internalize than Ad5 for human CD34+ cells, REVC, K562 and MCF-7 cells. This finding provides a basis for the construction of chimeric adenoviral vectors which are Ad5 vectors containing receptor ligands derived from other serotypes.

F. Infection studies on primary human bone marrow cells.

Since established erythroleukemic cell lines do not represent an adequate model for the ultimate hematopoietic stem cell that has to be targeted in patients in order to achieve long-term reconstitution with genetically modified cells, normal primary human bone marrow cells are used for the initial infection/retargeting studies.

RESULTS

In a first set of tropism studies with different Ad serotypes, whole bone marrow cell suspensions can be used without preselection. This is advantageous because the tropsim of various adenovirus serotypes or genetically retargeted vectors can be analyzed on a broad spectrum of progenitor subpopulations representing myeloid, erythroid, megakaryocytic, lymphoid, dentritic, and monocytic lineages. For short term (< 5 hours) infection studies, bone marrow suspensions can be cultured in IMDM supplemented with 10% FCS, β-mercaptoethanol, and 10u/ml IL-3 for ensuring cell viability.

Mononucleated cell assays:

Mononucleated bone marrow cells can be incubated with MOI 1, 10, 100, or 1000 pfu/cell of the various adenovirus types for a short time. Paraffin sections or cytospins of infected bone marrow cells can be analyzed for nuclear-localized, labeled viral DNA. BrdU labeling can be visualized by immunoflouresence with anti-BrdU antibodies; ^{32}P-tagged viral DNA can be detected by incubation with photo-emulsion. In addition, the same cell material can be analyzed for morphology after specific histo-staining (e.g. Wright-, Hemo3 staining). If required, commercially available antibodies can be used to specific cell surface markers conjugated directly to different fluorochromes (FITC (green), TRIT., RPE, (red), RPE-Cy5, AMCA (blue)) to completely characterize infected bone marrow subpopulations. Colocalization of BrdU-labeled viral DNA (e.g. as FITC signal) with membrane markers signifying infection of specific cell types can be demonstrated; for example, potential stem cells/early progenitors ($CD34^+$, $CD38^-$), megakaryocytes (CD41a+), eryhthroid cells (glycophorin A+), dentritic cells (CD1a+), monocytes (CD14+), or myeloid cells (CD15+), etc. The morphological analysis of infected bone marrow subsets gives a first information whether specific adenovirus serotypes can target primitive cell types.

DISCUSSION

Since the different wild-type adenoviruses do not express a uniform marker gene and do not integrate and since detection of tagged viral DNA cannot be done on live cells, it is not possible, at this point, to characterize infected cells for clonogenic or repopulation capacities. Therefore, adenovirus serotypes for retargeting studies are selected, based on their ability to infect in vitro purified CD34+ cells at low MOIs. This subset of bone marrow cells is known to contain long-term reconstituting cells. Infection studies with different adenovirus serotypes can be repeated on purified CD34+ cells (cultured in IMDM +10% FCS, β-mercaptoethanol, and 10 units/ml IL-3) as described above. Purification of CD34+ cells can be performed by direct immunoadherence on anti-CD34 monoclonal antibody-coated plates or on MiniMacs columns as described by

Papyannopoulou (Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69; Papayannopoulou, T. et al., 1993, *Blood*, 81:229). The purity of isolated CDC34+ cells ranges routinely from 80-95%. Analgous infection studies can be repeated with selected adenovirus types on CD34+/CD38- subsets.

To confirm productive infection purified CD34+ cells can be infected with selected (methylase-tagged) serotypes and analyze viral DNA replication. Cultures of purified human bone marrow CD34+ cells can be used for the transduction and integration studies as a model for HSCs.

It was recently demonstrated that HSC activity does exist in CD34-negative human bone marrow subsets (Bathia, M. et al., 1998, *Nature Medicine*, 4:1038-45; Osawa, M., et al., 1996, *Science*, 273:242-5; Goodell, M. et al., 1997, *Nature Medicine*, 3:1337-45; Zanjani, E. D. et al., 1998, *Exp. Hematology*, 26:353-60). $Lin^{-}CD34^{-}38^{-}$ cells can be tested in the retargeting and transduction studies in combination with repopulation assays in SCID-NOD mice.

<u>G. Cloning and insertion of the fiber gene.</u>

METHODS

<u>PCR-cloning of the corresponding fiber gene and insertion into Ad5 based shuttle plasmids instead of the endogenous AD5 fiber:</u>

One or several adenoviruses with tropism to CD34+ or other HSC containing population is selected for further studies described herein. The complete coding region for fiber varies between 1-2kb, depending on the virus type. The fiber encoding sequences can be obtained by PCR with Pfu polymerase from viral DNA isolated from purified particles of the selected virus types. The corresponding primers can be designed based on the fiber sequences available from the EMBL gene bank. The PCR products are cloned as PacI-BalI fragment into pCD4 (Figure 10), a shuttle vector for recombination of RecA+ E.

coli. In pCD4, the heterologous fiber gene is flanked on both sides with Ad5 sequences, which are homologous to regions directly adjacent to the fiber reading frame in Ad5. As an Ad5 (shuttle vector) derived template for recombination, pCD1, a pBHG 10 (Microbix, Toronto, Canada) derivative can be used. The recombination procedure is performed according to a protocol routinely used for recombinant adenovirus generation (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Routinely, 90% of the resulting plasmids are accurately recombined. The junctions between the heterologous fiber (X) and Ad5 sequences can be sequenced to confirm the accuracy of recombination. The resulting plasmid is named pAd5fiberX ($pAd5^{fx}$). The resulting product is used to generate $pAd5^{fx}$-based Ad.AAV containing the heterologous fiber gene.

Construction of chimeric Ad vectors:

For transduction studies, two Ad vectors were constructed: Ad5GFP and Ad5GFP/F35, containing a chimeric Ad5/35 fiber gene. Both adenoviral vectors contained a 2.3kb, CMV promoter driven EGFP gene [derived from pEGFP-1, (Clontech, Palo Alto, CA)] inserted into the E3 region of Ad5. The EGFP expression cassette was cloned between Ad5 sequences 25,191-28,191 and 30,818-32,507 into a shuttle plasmid, which contained the E3 deletion described for pBHG10 (Microbix, Toronto, Canada). The resulting plasmid was named pAdGFP. For the chimeric vector, the Ad5 fiber gene in pAdGFP was substituted by an Ad5/35 chimeric fiber gene generated by the two-step PCR protocol outlined above. In the first PCR step, three DNA fragments corresponding to i) the Ad5 fiber 5'-nontranslated region and the first 132 bp of the fiber tail domain (nt 30,818-31,174), ii) the Ad35 shaft and knob domains (nt 132-991), and iii) the Ad5 E4 region including the Ad5 fiber polyadenylation signal (nt 32,775-33,651 were amplified by Pfu-Turbo DNA polymerase. The following primers were used: for the Ad5 tail, Ad5F-2 (nt 30,798-30,825) 5'-CGC GAT ATC GAT TGG ATC CAT TAA CTA-3' and Ad5R-2 (nt 31,174-31,153) 5'-CAG GGG GAC TCT CTT GAA ACC CAT T-3'; for the Ad35 shaft and knob, primers Ad5/35F and Ad5/35R (see above); for the Ad5E4 and polyA, primers Ad5F-1 and Ad5R-1 (see above). After 10 PCR cycles, the products were purified by agarose gel electrophoresis, combined, and then subjected to a second PCR

with primers Ad5F-2 and Ad5R-1. The resulting 2115 bp-long chimeric fiber gene contained the Ad5 tail and the Ad35 shaft and knob domains. This product was used as a substitute for the SalI/XbaI Ad5 fiber gene containing fragment in pAdGFP. The resulting plasmid was named pAdGFP/F35. To generate full-length E1/E3 vector genomes, pAdGFP and pAdGFP/F35 were inserted in pAdHM4 (Mizuguchi, H., Kay, M.A. 1998. *Human Gene Therapy*. 9:2577-2583) by recombination in *E.coli* (Chartier, C., E. et al. 1996. *Journal of Virology*. 70:4805-4810). To do this, the RecA+ *E.coli* strain BJ5183 was co-transformed with pAdHM4 linearized by SrfI mixed with the XbaI fragments containing the GFP genes, the Ad5 or Ad5/35 fiber genes, and the Ad5 homology regions. The resulting recombinants were analyzed by restriction analysis. Correct recombinants were amplified in *E.coli* HB101 and purified by double CsCl gradient banding. The plasmids were named pAd5GFP and pAd5GFP/F35. The correct structure of the Ad5/35 chimeric fiber gene was confirmed by endonuclease digestion and sequencing part of pAd5GFP/F35. To produce the corresponding viruses, pAd5GFP and pAd5GFP/F35 were digested with PacI to release the viral genomes and transfected onto 293 cells as described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaques developed 7 to 10 days post-transfection in overlayed cultures. Recombinant viruses were propagated in 293 cells and purified by standard methods described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960).

Hemagglutination assay:

Twenty-five microliters of serial dilutions of Ad5, Ad35, or chimeric Ad5GFP/F35 virions in McIlvaine-NaCl buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 [pH 7.2], diluted 1:50 with 0.87% NaCl) were loaded onto 96 well plates. To each dilution, 25 μl of a 1% suspension of monkey erythrocytes (in McIlvaine-NaCl buffer) was added. The sedimentation pattern was determined after incubation for 1 hour at 37°C. All tests were performed in quadruplicates in at least two independent experiments.

Southern blot:

Extraction of genomic DNA, labeling of DNA fragments and hybridization were performed as described earlier (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960).

RESULTS

Construction/Characterization of chimeric fiber:

Previously, it has been shown that exchanging the fiber knob was sufficient to alter the tropism of chimeric Ad vectors (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540; Krasnykh, V., et al. 1998. *J. Virology*. 72:1844-1852; Stevenson, S. C., et al. 1997. *J. Virology*. 71:4782-4790). As outlined above, the length of the fiber shaft may critically determine the entry strategy of a particular serotype. Therefore, we decided to replace not only the Ad5 fiber knob but also the shaft. The chimeric Ad5/35 fiber contained the Ad5 tail (amino acid: 1-44) necessary for interaction with the Ad5 penton base linked to 279 amino acids from Ad35 including the shaft with 7 β-sheets and the knob (Fig. 16A). The endogenous Ad5 fiber polyA signal was used to terminate transcription of the chimeric fiber RNA. The combination of the Ad5 capsid including the RGD motif containing penton base with a short-shafted fiber could be risky because the natural distance between the fiber knob and the RGD motifs was disturbed. The Ad5 fiber was substituted by the chimeric fiber sequences based on an E1/E3 deleted Ad vector. This vector carried a CMV promoter-GFP reporter gene cassette inserted into the E3 region. The corresponding chimeric virus (Ad5GFP/F35) was produced in 293 cells at a titer of $>2x10^{12}$ genomes per ml. For comparison, an E1/E3 deleted Ad vector containing the original Ad5 fiber gene and the GFP expression cassette was generated (Ad5GFP). The titer and the ratio of physical to infectious particles was similar between Ad5GFP and Ad5GFP/F35 indicating that the fiber modification did not significantly alter the stability and/or growth properties of the chimeric vector. The correctness of the fiber modification was confirmed by restriction analysis of the Ad5GFP/F35 viral genome followed by

Southern blot hybridization (Fig. 16B), direct sequencing of the fiber-coding region, and a functional test for hemagglutination (HA) of monkey erythrocytes. The agglutination of erythrocytes is fiber knob-mediated; it is known that Ad5 does not agglutinate monkey erythrocytes whereas Ad35 efficiently does (Pring-Akerblom, P., et al. 1998. *J. Virology.* 72:2297-2304). In HA tests, Ad5GFP/F35 agglutinated monkey erythrocytes with the same efficiency as Ad35 at dilutions of up to 1:512. In contrast, no hemagglutination was observed with equivalent Ad5 dilutions. This clearly confirmed the functional activity of the chimeric Ad5/35 fiber incorporated into Ad5 capsid.

Generation of chimeric adenoviral vectors (Ad.AAV^{fx}) with heterologous fiber molecules: Adenoviruses with chimeric Ad5-Ad3 fiber are viable and can be produced at high titers (Krasnykh, V., et al., 1996, *J. of Virology*, 70, 6839-6846; Stevenson, S. C. et al., 1997, *J. Virology*, 71:4782-90). In order to test whether the fiber substitution described herein affects production or stability of adenoviruses, two E1-deleted first-generation, adenoviral vectors are produced with the AAV-□gal cassette in 293 cells using standard protocols. The vector is generated by recombination of pAd.AAV-BG (Fig. 17) with pCD1 (containing the endogenous Ad5 fiber); the other vector (with heterologous fiber) is the recombination product of pAd.AAV βGal and pAd5fiberX (pAd5^{fx}). Virus from single plaques is amplified on 293 cells. The production yield per 293 cell can be determined by plaque-titering of 293 cell Lysates. It is anticipated that the fiber modification will not critically affect the stability of chimeric vectors. Finally, bone marrow cells can be infected with the retargeted vectors. Two days after infection, live-cell cytometry is performed for β-gal expression using as substrate Fluorescein di-□-D-Galactopyranoside (FDG) (Cantwell, M.J. et al., 1996 *Blood* 88, 4676-4683; Neering, S. et al., 1996, *Blood*, 88:1147-55; Fiering, S. N. et al., 1991, *Cytometry*, 12:291; Mohler, W. et al., 1996, *PNAS*, 93:57) and the infected cells are characterized for morphology and surface markers. Before and during infection, bone marrow cells can be cultured in IMDM/FCS supplemented with thrombopoietin (Tpo), which supports the survival of HSC (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). Alternatively, retargeted vectors can be

generated with the AAV-GFP (green fluorescence protein) cassette and perform FACS analysis on transduced cells based on GFP and surface marker expression.

H. Competition studies of chimeric fiber protein Ad5/35.

Competition studies:

Cross-competition studies between Ad5, 35, and Ad5GFP/F35 (Fig. 18) for binding and internalization were performed in order to investigate in more detail the pathways which are used by the chimeric vector to infect target cells. Wild-type Ad35 and the chimeric vector Ad5GFP/F35 could recognize the same primary receptor as they competed with each other for the attachment to K562 cells (Fig. 19A, upper panel). This primary receptor is different from that used by Ad5, since neither Ad5 viral particles nor anti-CAR monoclonal antibodies (Fig. 19B, upper panel) were able to abrogate Ad35 or Ad5GFP/F35 binding. In competition studies for internalization, Ad35 and Ad5GFP/F35 competed with each other with equal efficiency. Ad5 and anti-α_v-integrin monoclonal antibodies (L230) (Figs. 19C, D; lower panel) did not inhibit internalization of Ad35 or the chimeric virus. To consolidate this data, K562 cells were infected with Ad5GFP and Ad5GFP/F35 after prior incubation of cells with anti-CAR or anti-α_v-integrins monoclonal antibodies followed by analysis of GFP-expressing cells. The transduction data mirror the results obtained in adsorption/internalization studies. In summary, this demonstrated that Ad35 and Ad5GFP/F35 use a CAR and α_v-integrin-independent pathway for infection of K562 cells; the structural elements which account for these specific properties are located within the Ad35 fiber and can be transplanted into Ad5 by fiber substitution.

Ad3 can efficiently interact with K562 cells (Fig. 12), although Ad3 and Ad35 belong to the same subgroup (B), the homology between amino acid sequences of their fibers is only about 60%. Therefore, we decided to test whether Ad3 could compete with Ad35 and Ad5GFP/F35 for attachment and internalization (Fig. 20). These studies demonstrated that Ad35 binding was not inhibited by Ad3 indicating the use of different

receptors. Interestingly, Ad3 slightly inhibited attachment of Ad5GFP/F35 (Fig. 20A, left panel). In addition to binding to the receptor common for the Ad35 and Ad5GFP/F35 fiber, the chimeric capsid (e.g. the Ad5 penton RGD motifs) may also interacts with a second cellular receptor that overlaps with elements involved in Ad3 binding. In cross-competition for internalization, pre-incubation of cells at 37°C with Ad35 and with chimeric virus significantly decreased internalization of [^{3}H]-labeled Ad3 (Fig. 20D, right panel). In the reverse experiment, Ad3 as competitor decreased the level of internalization by 30% for both, Ad35 and the chimeric virus (Fig. 20B, right panel). As expected, Ad5 and Ad3 did not compete for adsorption or internalization. As shown before (Fig. 19B), anti-CAR and anti-α_v-integrin antibodies did not block Ad3 interaction with K652 cells. In summary, we concluded that Ad35 and Ad5GFP/F35 bind to receptor/s different from that of Ad3, although they can use common structural elements for internalization, which are different from α_v-integrins.

Infection studies with chimeric virus:

It is established that Ad5GFP/F35 infected K562 cells by a CAR and α_v-independent pathway. It is possible that this property allows for efficient transduction of non-cycling CD34+ cells, which express scarcely CAR and α_v-integrins. To test this, the transduction properties of Ad5GFP and Ad5GFP/F35 vectors were analyzed on CD34+ cells, K562, and HeLa cells. Fig. 21 shows the percentage of transduced, GFP expressing cells depending on the MOI used for infection. Nearly 100% of HeLa cells were transduced with Ad5GFP and Ad5GFP/F35 at MOIs of >/=25. More than 95% of the K562 cells were transduced with Ad5GFP/F35 at MOIs of >/= 100, whereas the transduction rate was significantly lower with Ad5 where it increased with the MOI reaching a plateau at ~70% GFP-positive cells after infection with an MOI of 400. Transduction of CD34+ cells was about three fold more efficient with Ad5GFP/F35 than with Ad5GFP at all MOIs analyzed. Interestingly, at higher MOIs, the transduction rate did not rise proportionally with the viral dose and soon reached a plateau indicating that in both cases only specific subset/s of CD34+ cells were permissive to infection. In order to characterize in more detail these specific, permissive subset/s, additional transduction

studies were performed. First, the percentage of GFP expressing cells was determined in CD34+ fractions that were stained for α_v-integrins or CARs (Fig. 22). The low number of CAR positive CD34+ cells complicated accurate co-labeling studies, and there was no correlation between CAR expression and the proportion of transduced cells among CD34+ cells infected with Ad5GFP or Ad5GFP/F35. Interestingly, for Ad5GFP, 65% of all GFP expressing cells were positive for α_v-integrins, whereas less than 22% of GFP positive cells infected with the chimeric virus stained positive for □$_v$-integrin expression. While only 17% of the whole CD34+ population expressed GFP after Ad5GFP infection, the percentage of GFP–expressing cells in the CD34+/ □$_v$-integrins positive fraction was 50%. This indicates that Ad5GFP vector-mediated GFP expression was preferentially localized to α_v-integrin positive CD34+ subsets, whereas after infection with the Ad5GFP/F35 vector, GFP was expressed in a broader spectrum of CD34+ cells with most of them being α_v-integrin-negative.

Next, transduced cells were simultaneously analyzed for GFP as well as for CD34 and CD117 markers. As mentioned before, only about 90% of all cells used in our analysis were positive for CD34 at the time of infection, hence the multiparameter analysis for CD34 and GFP. A population of CD34+ cells is extraordinarily heterogeneous in morphology and stem cell capacity. The subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (*Ikuta, K., Weissman, I.L. 1992* Proc. Natl. Acad. Sci. USA. *89:1502-1506*; *Simmons, P. J., et al. 1994.* Expl. Hematology. *22:157-165*). Fig. 23 summarizes the analyses of GFP expression in correlation with these specific stem cell markers. While 54% of cells infected with chimeric vector were positive for GFP and CD34+, only 25% of cells infected with Ad5GFP expressed the transgene and CD34+ marker (Fig. 23A, lower panel). More importantly, based on GFP expression, the chimeric virus transduced 80% of c-kit positive cells, whereas the Ad5-based vector transduced only 36% (Fig. 23A, middle panel). In an additional experiment, CD34+ cells were sorted for CD117 expression prior to infection with Ad5GFP or Ad5GFP/F35 and, 24 hours post-infection, GFP expression was analyzed in this specific fraction (Fig. 23B). This analysis revealed that the chimeric vectors transduced 4 fold more CD34+/CD117+ than the Ad5GFP vector.

In conclusion, these results demonstrated that the chimeric Ad5GFP/F35 vector was clearly superior to the Ad5GFP vector in targeting and transduction of CD34+ cells. Furthermore, the data suggest that the spectrum of CD34+ cell subsets permissive for Ad infection was significantly different for the chimeric vector than for the Ad5 vector.

Analysis of viral genomes within CD34+ cells infected with the Ad5 and chimeric vectors:

So far, the transduction rate of CD34+ cells was measured based on GFP expression after infection with Ad5GFP and Ad5GFP/F35. Considering the extraordinary heterogeneity of CD34+ cells in morphological and functional parameters, GFP may not be expressed in all cell types that were efficiently infected. Reasons for this include that the CMV promoter may not be active in all cell types or that the regulation of transgene expression could differ between subsets on a post-transcriptional or post-translational level. To test this, we quantified the number of intracellular (transduced) viral genomes within GFP positive and GFP negative fractions of CD34+ cells infected with Ad5GFP and Ad5GFP/F35. To do this, twenty-four hours after infection, CD34+ cells were sorted for GFP positive and GFP negative fractions, which were subsequently used to isolate genomic DNA together with transduced viral DNA. The number of viral genomes was determined by quantitative Southern blot as described for Fig. 15. Per GFP-positive CD34+ cell, about 270 copies of the Ad5GFP/F35 viral genome were detected. Interestingly, a remarkable 200 copies of the Ad5GFP/F35 viral genome were found per GFP-negative CD34+ cell (Fig. 24A and 25). This demonstrated that not all infected cells expressed GFP and implies that the actual transduction rate was higher than 54% (GFP-positive cells). We concluded that the CMV promoter was not active in all transduced CD34+ subsets. No Ad5GFP vector specific signal was detected within infected CD34+ (GFP positive or negative) fractions by Southern blot which had a detection limit of 14 viral genomes per cell. From this, we can conclude that the vector DNA concentration per transduced cell was at least 20 times higher for Ad5GFP/F35 than for Ad5GFP.

Ad5GFP DNA was only detectable in DNA samples from infected CD34+ cells by Southern blot after prior PCR amplification with vector specific primers (Fig. 24B and 25). This indicates that the replication deficient Ad5 vector is present but at a very low copy number, which may be limited by intracellular genome stability. Using the PCR-Southern detection method, Ad5 vector DNA was also detected in GFP negative cells, supporting that the CMV promoter may not have been the optimal choice for transduction studies. It is notable that studies by others on viral genome analyses after infection of CD34+ cells with Ad5 vectors were performed only after prior PCR amplification (Mitani, K., et al. 1994. *Human Gene Therapy.* 5:941-948; Neering, S. J., et al. 1996.. *Blood.* 88:1147-1155).

DISCUSSION

The chimeric Ad5GFP/F35 vector has binding and internalization properties similar to Ad35. Therefore, the fiber substitution was sufficient to swap cell tropism from Ad5 to Ad35. The Ad5GFP/F35 capsid chimera contained the short-shafted Ad35 fiber incorporated into an Ad5 capsid, instead of the naturally occurring long-shafted Ad5 fiber. During Ad5 infection, interaction between the penton base and intergrins is required to induce viral internalization. For this interaction, the length of fiber shaft and the precise spatial arrangement of knob and RGD motifs are critical for the virus entry strategy. The natural spatial arrangement is disturbed when short-shafted heterologous fibers are inserted into the Ad5 capsid. Interestingly, the Ad5/35 capsid chimera allows for efficient infection, suggesting that the protruding RGD motives in the Ad5 penton base do not affect the interaction with the primary Ad35 receptor. So far, most of the chimeric viruses were generated by substituting only the Ad5 knob while maintaining the long Ad5 fiber shaft (Chillon, M., et al. 1999. *J. Virology.* 73:2537-2540; Krasnykh, V. N., et al. 1996. *J.Virology.* 70:6839-6846; Stevenson, S. C., et al. 1995. *J. Virology.* 69:2850-2857; Stevenson, S. C., et al. 1997. *J.Virology.* 71:4782-4790). The exception was an Ad5/7 chimeric virus (Gall, J., et al. 1996. *J. Virology.* 70:2116-2123), where the whole Ad5 fiber was substituted by the short-shafted Ad7 fiber. However, similar to the parental Ad5, the Ad5/7 chimera still required α_v-integrins for infection.

This Ad5GFP/F35 chimera is the first demonstration that despite the presence of RGD motifs within the Ad5 penton, the chimeric virus uses cell entry pathways determined primarily by the receptor specificity of the short-shafted heterologous fiber. This does not exclude that interaction with a secondary receptor may increase binding affinity. The latter is supported by the observation that Ad35 and Ad5GFP/F35 slightly differed in their ability to compete with Ad5 or Ad3 for binding. It is possible that Ad5/35 attachment involves, in addition to the high affinity fiber binding, interaction between Ad5 capsid proteins (e.g. RGD motifs) and secondary receptor/s that overlap with those used by Ad3 and Ad5.

This data indicate that infection with Ad5-based vectors is restricted to a specific subset of CD34+ cells. The percentage of GFP expressing cells after Ad5GFP infection of CD34+ cells reached a plateau at MOIs higher than 100 indicating that only a limited fraction of CD34+ cells was permissive to Ad5. Also, strong replication of wild type Ad5 in infected CD34+ cells may be the result of preferential transduction of a specific subpopulation of CD34+ resulting in a expression of early viral genes at a level sufficient to initiate viral replication. The presence of a specific subpopulation of CD34+ cells permissive to Ad5-vector infection was suggested by others (Byk, T., et al. 1998. *Human Gene Therapy*. 9:2493-2502; Neering, S. J., et al. 1996.. *Blood*. 88:1147-1155). In the present report, we further characterized this subpopulation and demonstrated that Ad5-based vectors preferentially infected α_v-integrin positive CD34+ cells. Integrins (includingα_v) are thought to be important for homing and trafficking of transplanted hematopoietic cells, however little is known about the correlation between α_v-integrin expression and the differentiation status of hematopoietic cells (Papayannopoulou, T., Craddock, C. 1997. *Acta Haematol.* 97:97-104; Roy, V., Verfaillie, C.M. 1999. *Exp. Hematol.* 27:302-312). There was no clear correlation between CAR and GFP expression suggesting that Ad5GFP may be able to use another membrane protein as a primary receptor. Alternatively, Ad5GFP transduction observed at an MOI of 200-400 could be the result of direct interaction between virus and α_v-integrins triggering internalization, which may be the preferred pathway in the absence of CAR (Legrand, V., et al. 1999. *J.*

Virology. 73:907-919). Importantly, infection with the chimeric Ad5GFP/F35 vector was not restricted to the α_v-positive CD34+ subpopulation.

Among CD34+ cells, the subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). The receptor for stem cell factor, CD117 (c-kit) belongs to a tyrosine kinase family. It was previously shown that c-kit+, CD34+ cord blood cells contain a high fraction (16%) of hematopoietic progenitors (Neu, S., et al. 1996. *Leukemia Research*. 20:960-971). Early in ontogeny 34+/CD117+ cells have long-term repopulating activity (Sanchez, M. J., et al. 1996. *Immunity*. 5:513-525). An average of 50-60% of CD34+ cells are reported to be CD117 positive (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Neu, S., et al. 1996. *Leukemia Research*. 20:960-971; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). In our studies, the chimeric vector expressed GFP in 54% CD34+ cells and 80% of CD34+/c-kit+ cells. The actual viral transduction rate could be even higher because transduced Ad5GFP/F35 vector DNA was also found in GFP-negative fractions of infected cells. This indicates that the CMV promoter used to drive GFP expression in our vectors was not active in all transduced cells. We selected the CMV promoter for transgene expression based on published data demonstrating that PGK and CMV promoters allowed for efficient transgene expression in CD34 cells whereas the HTLV-I and RSV promoter were almost inactive (Byk, T., et al. 1998. *Human Gene Therapy*. 9:2493-2502; Case, S. S., et al. 1999. *Proc. Natl. Acad. Sci. USA*. 96:2988-2993). On the other hand, studies by Watanabe et al. (Watanabe, T., et al. 1996. *Blood*. 87:5032-5039) suggest that the CMV promoter is not active or rapidly silenced in certain CD34+ subsets. Our data underscore this observation. Considering retroviral transduction studies, the retroviral MLV promoter may have been a better candidate for transduction studies in hematopoietic cells (Bregni, M., et al. 1998. *Gene Therapy*. 5:465-472).

After having demonstrated that the Ad5GFP/F35 vector efficiently transduced cells carrying stem cell specific markers, the next logical step would be to perform colony

assays with pre-sorted GFP positive/negative cells. However, this assay is complicated by the fact that infection with first generation Ad vectors is cytotoxic and affects the formation and growth of progenitor colonies in MC-cultures (Mitani, K., et al. 1994. *Human Gene Therapy*. 5:941-948; Watanabe, T., et al. 1996. *Blood*. 87:5032-5039). This side effect is caused by the expression of Ad proteins within transduced cells (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960; Schiedner, G., et al. 1998. *Nature Genetics*. 18:180-183; Yang, Y., et al. 1994. *Proc. Natl. Acad. Sci. USA*. 91:4407-4411). Some of these proteins (e.g E4-orf4, pTP, or E3-11.6k) have pro-apoptotic activity (Langer, S. J., Schaak, J. 1996. *Virology*. 221:172-179; Lieber, A., et al. 1998. *J. Virology*. 72:9267-9277; Shtrichman, R., Kleinberger, T. 1998. *J. Virology*. 72:2975-2983; Tollefson, A. E., A et al. 1996 *J. Virology*. 70:2296-2306). Clearly, this would affect the outcome of transduction studies with Ad5GFP/F35, which allows for the efficient transfer of viral genomes into CD34+ cells implying significant expression of viral proteins. Moreover, recently published data indicate that short-term colony assay mostly measure mature progenitors and do not represent a rigorous test for transduction of potential stem cells.

A definitive demonstration that Ad5GFP/F35 based vectors can transduce HSC requires colony assays or preferably, repopulation assays in SCID-NOD mice. We can perform these studies with gutless vectors (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) and integrating □Ad.AAV vectors devoid of all viral genes (Lieber, A., et al. 1999. *J Virol* 73:9314-24) generated based on Ad5GFP/F35 chimeric capsids. Alternatively, gutless, retargeted vectors could be used to transiently express a retroviral receptor on CD34+ cells to increase their susceptibility to infection with retroviral vectors based on an approach that we have published earlier (Lieber, A., et al.. 1995. *Human Gene Therapy*. 6:5-11).

Our finding that Ad5GFP/F35 can efficiently transduce hematopoietic cells with potential stem cell capacity represents an important step towards stable gene transfer into HSCs and gene therapy of blood disorders. Furthermore, the virological aspects of this invention contribute to a better understanding of adenovirus cell interactions.

I: Retargeting of Ad5 based vectors with modified fibers carrying specific ligand peptides for HSC and other cell types

Another alternative to make Ad5-capsid-based vectors suitable for HSC gene therapy is to incorporate the coding sequence for HSC specific peptides into the H1 loop region of the Ad5 fiber gene. The modification of the H1-loop was successfully exercised by Krasnykh et al. with a 7 amino-acid long FLAG peptide (DYDDDDK). Using phage display peptide libraries (Pascqualini, R. et al., 1996, *Nature*, 380:364-66), Renata Pasqualini (La Jolla Cancer Research Center) reported recently, at the First Meeting of the American Society for Gene Therapy, the identification of small peptide ligands specific for bone marrow cells. The corresponding sequences encoding these peptides can be added to modify the H1 loop sequence employing site-directed mutagenesis. Optimally, the ligands should allow for the efficient internalization of adenoviral particles based on a CAR- and integrin independent pathway. Modified adenoviral vectors containing the AAVBG cassette can be produced and tested for HSC tropism as described above.

Adenovirus peptide display:

In order to retarget adenoviruses to any cell type of interest, a strategy is provided which involves creating a library of adenoviruses displaying random peptides in their fiber knobs as ligands and screening this library for adenovirus variants with tropism to a particular cell type in vitro and potentially in vivo.

The development of the adenovirus peptide display technique is based on the following ideas. (i) Although the tertiary structure of the Ad5 fiber knob is known, it remains unclear which domains are involved in receptor binding. There are data suggesting that receptor-binding domains partially overlap with hemagglutination domains, which are well characterized for a number of serotypes. Therefore, three intramolecular loop regions representing potential receptor binding sites can be substituted by random peptide

libraries. Eight amino acid residues in the center of the FG, or GH loops can be substituted by octameric random peptides (Figs. 26 and 27). These substitutions will replace CAR tropism and allow for infection of refractory cell types. (ii) To synthesize the oligonucleotides encoding the peptide library a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids is employed. This avoids termination codons and assures optimal codon usage and translation in human cells. Synthesis of a completely randomized library is possible with all 20 amino acids being incorporated with the same probability and a partially randomized library with only three (in average) random amino acids substitutions per octamer at random positions with a random amino acid to maintain certain critical features of the tertiary knob structure while introducing variability. The last model is based on the distribution of amino acids present in the hypervariable CDR 1 or 2 region of immunoglobulins. (iii) To maintain a representative library size of about 10^{10} different octamers per modified loop, a new cloning strategy is employed to allow for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. This strategy is based on a "seamless" cloning technique available from Stratagen. (iv) In order to produce the library of viruses, viral genomic DNA containing the modified fiber sequences is transfected into 293 cells without reduction of the library size. This critical step is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via poly-lysine to assure 100% transfection efficiency. This technique allows for coupling of ~1 μg of plasmid DNA (or ~1×10^{10} adenoviral genomes) to 10^{10} viral particles which can be used to infect 293/cre cells at an MOI of 10-100. Importantly, the carrier adenoviral genome has the packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express cre recombinase (293/cre). This helper virus system is routinely used to produce so-called gutless adenoviruses. Therefore, the virus progeny represents library genomes packaged into capsids containing preferentially Ad5 fibers. This is important for the next infection step into 293 cells at a MOI of 1 to assure a homogeneous fiber population on the capsid where the fibers are encoded by the packaged genome.

J. Production Of Adenovirus Vectors With Increased Tropism To Hepatocytes

An example of a G-H loop substitution to target Ad5 to hepacytes was successful. Preliminary tests demonstrated that two evolutionarily conserved regions within the malaria circumsporozoite surface protein (CS) termed RI and RII+ mediate specific interaction with hepatocytes but not with other organs (including spleen, lung, heart and brain), nor with Kupffer cells, liver endothelial cells or with other regions of the hepatocyte membrane (Cerami, C. et al., 1992, *Cell,* 70:1021-33; Shakibaei, M. and U. Frevert, 1996, *J. Exp. Med.,* 184:1699-711). These regions are conserved among different species including Plasmodium berghei, P. cynomogli, and P. falciparum that infect mouse, monkey and human hepatocytes, respectively (Cerami, C. et al., 1992, *Cell,* 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81). Peptides derived from RI (KLKQPG) or RII (EWSPCSVTCGNGIQVRIK) blocked CS binding to hepatocytes and infection by sporozoites in vivo ((Cerami, C. et al., 1992, *Cell,* 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81). RI and RII+ peptides were separately inserted into Ad5-fiber knob (H-I and G-H loop) containing mutation with abolished binding to CAR and alpha-v integrins (Kirby, L. et al., 2000, *J. Virol.,* 74:2804-13; Wickham, T. J. et al., 1995, *Gene Ther.,* 2:750-6). Based on preliminary data, a short-shafted fiber was used so that the virus entry strategy predominantly depends on the interaction with the primary (hepatocyte-specific receptor). The hepatocyte-specific ligands are flanked by short glycine stretches to provide flexibility and embedded into a loop formed by two cystines. This is one of the classical strategies to incorporate ligands into a protein scaffold (Doi, N. and H. Yanagawa, 1998, *Cell Mol. Life Sci.,* 54:394-404; Koivunen, E. et al., 1995, *Biotechnology (NY),* 13:265-70) and to guarantee their presentation at the protein surface. The biodistribution of the best variants is tested in vivo in C57B1/6 mice based on Southern blots or PCR for vector DNA in different organs. This mouse strain in known to be susceptible to infection with *P. berghei* (Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81).

K. Production Of Adenovirus Vectors With Increased Tropism To Tumor Cells

A similar strategy is to insert two peptides obtained after selection for tumor tropism by displaying random peptides on filamentous phages. The first double cyclic peptide (RGD-4) proved to bind specifically to integrins present on tumor vasculature (Ellerby H. M. et al., 1999, *Nat. Med.*, 5:1032-8). The second peptide targets specific matrix metalloproteinases associated with metastatic tumor cells as shown for the breast cancer cell line MDA-MB-435 (Koivunen, E. et al., 1999, *Nat. Biotechnol.*, 17:768-74). Tropism-modified vectors are tested in animal models with hepatic metastases derived from MDA-MB-435 cells (Fig. 28).

L. Development Of A Peptide Display Technique Based On Adenoviruses

A synthetic peptide library is described that allows adenovirus vectors to express random peptides in the G-H loop of the fiber knob domain. The technique of a phage display library is optimized to generate a library of adenoviruses displaying random peptides in their fiber knob. This library of adenovirus variants is then screened for tropism to a particular cell type in vitro and potentially in vivo. The oligonucleotides encoding the peptide library employ a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids. This will end the termination codons and assure optimal codon usage and translation in human cells. To maintain a representative library size, a new "seamless" cloning strategy that allows for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. Transfection into 293 cells is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via polylysine to assure a 100% transfection efficiency. Importantly, the carrier adenoviral genome has its packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express Cre recombinase (293/cre). The library is produced with E1-positive viruses depleted for CAR and integrin tropism. Only variant that have successfully infected the cell type of interest will replicate, resulting in de novo produced

virus. The sequence of the peptide ligand that conferred the particular tropism will then be analyzed in do novo produced virus.

EXAMPLE III

COMBINATION NOVEL ADENOVIRAL VECTOR AND MODIFIED FIBER PROTEIN

This example describes the following studies which combine the technology of the integrating adenovirus vector that is devoid of all adenoviral genes with the modified fiber protein that retargets the vector to quiescent HSC.

A. Transduction studies with re-targeted vectors in HSC:

In order to transduce quiescent HSC and integrate into chromosomal DNA, retargeted ΔAd.AAV^{fx} vectors are tested for reporter gene expression, and vector integration simultaneously while analyzing their clonogenic capacity. The modified ΔAd.AAV^{fx} hybrid vectors contain genomes devoid of all adenoviral genes (a "gutless" adenovirus vector) packaged into Ad5 capsids with modified fibers. Rep may be incorporated into these ΔAd.AAV^{fx} vectors to allow for site-specific integration into AAVS1.

Transduction studies:

Purified human CD34+ cells in IMDM/FCS+IL-3 and SCF are infected with different doses of ΔAd.AAV^{fx}-BG (1-10^7 genomes per cell). CD34+ cells infected with ΔAd.AAV^{fx}-βGal are cultured for 2 days in suspension and sort β-Gal+ cells by FACS using FDG as substrate. This determines the infection efficiency. β-gal expressing cells are then submitted to clonogenic assays in semi-solid cultures (in two dishes per MOI) in the presence of multiple cytokines. (IL-3, SCF, Epo, G-CSF, GM-CSF, IL-7, Tpo). A first set of semi-solid cultures can be evaluated after 7 days; another set can be analyzed after 14 days. Colonies that have formed in semisolid culture can be characterized by

light microscopy and subsequently stained with X-Gal staining. Most of the vector genomes should remain episomal and can be lost with successive cell divisions. Thus, while most cells can be X-Gal positive at day 2 or day 7 after infection, most of the larger colonies (analyzed at day 14 p.i.) may not stain homogeneously for β-Gal. A representative number of X-Gal positive and X-Gal negative colonies can be picked and analyzed for episomal and integrated vector DNA. The outcome depends on the MOI used for infection and the integration status of the vector. These studies determine whether hybrid vectors can infect primitive progenitors.

Detailed characterization of hybrid vector integration:

CD34+ cells can be infected with ΔAd.AAV^{fx}-SNori (MOI 1-10^{7}) and subjected to G418 selection in methyl cellulose (MC) cultures in the presence of growth factors (IL-3 and SCF). The resulting colonies are a mixture of mainly myeloid cells. The number and morphology of G418 resistant colonies can be determined after 2 weeks of selection. This strategy may be disadvantageous in that the appropriate stem cell may not divide and form G418 resistant colonies under the specific culture condition used. Moreover, it may be difficult to perform G418 selection on a population of heterogenous cells, which vary in their sensitivity to G418. Therefore, another set of ΔAd.AAV^{fx}-SNori infected CD34+ cells can be cultured in methyl-cellulose (+IL-3, SCF) without G418 selection. After 2-3 weeks, single colonies can be picked from both (w/ and w/o G418) MC cultures, morphologically characterized, and analyzed for integrated vector using the modified protocol developed for integration studies in a small number of cells (see Figure 8). This strategy allows the assessment of whether hybrid vectors integrate into the genome of CD34+ cells cultured in the presence of growth factors. This study gives us an idea about potential position effects affecting neo or βgal expression from integrated vector copies and about the structure of the integrated vector and the flanking chromosomal regions.

An alternative method to confirm vector integration:

Fluorescence *in situ* hybridization (FISH) analysis, can be performed in individual cells from MC colonies. CD34+ cells are cultured in MC in the presence of growth factors to induce cell division and subsequently treated with colchicine. Metaphase chromosome spreads are analyzed with biotin-ATP labeled probe specific for the βGal or SNori gene and a dioxigenin-UTP labeled probe for the human X-chromosome as an internal control (provided by Christine Disteche, University of Washington). Specific hybridization can be visualized with corresponding anti-biotin or anti-DIG antibodies labeled with different fluorochromes (e.g. FITC and Texas Red). Hybrid vector DNA may integrate as concatemers, which would facilitate detection by FISH. This technique allows one to localize the chromosomal integration sites of hybrid vectors.

Test transduction into quiescent bone marrow subpopulations:

Hybrid vectors described so far can be tested to see whether quiescent CD34+ cells can be stably transduced. To avoid significant cell proliferation, purified CD34+ cells are cultured in serum free IMDM supplemented with thrombopoietin (Tpo). Tpo can alone support the survival of stem cells without stimulating their active cell proliferation (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). To analyze the proliferation status of CD34+ cells at the time point of infection with the hybrid vector ΔAd.AAV^{fx}BG, BrdU is added 2 hours before infection to the culture medium. One set of cells are maintained as suspension culture in IDAM containing Tpo only for two days. Another set of cells are grown in IDAM+Tpo supplemented with multiple cytokines. Forty eight hours after infection, CD34+ cells can be FACS sorted for beta Gal expression using FDG. FDG positive cells can be further analyzed for cellular DNA replication based on BrdU incorporation and for specific CD34+ subset markers. To do this, cytospins from FDG+ cells can be submitted to immunofluorescence with BrdU specific antibodies and with antibodies to specific cell surface markers (e.g. CD38, CD41). Alternatively, consecutive paraffin sections of the same cell can be analyzed for (a) transgene expression by X-Gal staining, (b) DNA synthesis based on BrdU incorporation, and (c) specific surface markers. This allows one to confirm that the culture conditions with Tpo alone prevent

significant genomic DNA replication and subsequent cell proliferation as well as to determine whether quiescent CD34+ cells can be infected based on beta Gal expression in cells where BrdU labeling is absent.

Test hybrid vectors integration into quiescent CD34+ cells:

Two sets of CD34+ cells are infected. The first set of □Ad.AAV^{fx}SNori infected cells are cultured for 5-7 days in the presence of cytokines; the other set is cultured without cytokines. To maintain CD34+ cell viability without cytokines during this period, the cells are cultured in the presence of Tpo or underlaid with a stromal cell line (AFT024) (Moore, K. et al., 1997, *Blood*, 89:4337-47), which can maintain HSC viable for 4 to 7 weeks. After this specific time period, both sets are submitted to clonogenic assays (in the presence of multiple cytokines) either in combination with G418 selection or without selection. Single colonies are analyzed morphologically and submitted to genomic DNA analysis (Figure 8) to determine the vector integration status. The ultimate proof for stem cell transduction is the in vivo survival/expansion assay. To do this, the CD34+ cells expressing beta Gal are used for transplantation experiments, If the number of FDG+ cells is not sufficient, total ΔAd.AAV^{fx}BG infected cells as well as all ΔAd.AAV^{fx}-SNori infected cells can be used directly without selection. Transplantation can be performed via tail vein injection into sublethally irradiated SCID NOD mice (Dao, M.A., et al., 1998, *Blood*, 4, 1243-1255; Matsunaga, T. et al., 1998, *Blood*, 92:452-61). At different time points after transplantation (4 to 8 weeks), mice can be sacrificed to obtain bone marrow cells which then can be cultured in suspension until various assays are performed for X-Gal and cell markers as described earlier. These cells also can be submitted to a secondary colony assay in MC or secondary transplantation into SCID NOD mice. Furthermore, MC colonies derived from these cells can be analyzed for the presence of integrated vector DNA by the method illustrated in Figure 8. The expression and integration data together allow conclusions about the repopulation efficiency and about potential position effects.

B. Optimization of ΔAd.AAV^{fx} vectors for γ r-globin expression in hematopoietic stem cells:

One specific example of the invention is (a) to construct retargeted hybrid vector with the γ -globin as the transgene under the control of erythroid cell specific promoter, (b) to analyze the level and kinetics of γ -globin expression after transduction with hybrid vectors in *in vitro* and *in vivo* assays, (c) if required, to protect gene expression from position effects using γ -globin LCRs or insulators incorporated into hybrid vectors, and (d) to study whether γ -globin introns or heterologous introns can increase γ -globin expression.

Another central issue of the invention is to demonstrate that hybrid vectors can accommodate larger transgenes than rAAV and retroviruses. The insert size limitation of these vectors is 5kb. Transgene cassettes up to 8kb can be inserted into hybrid vectors as described. The maximal insert size may be about 14kb, if hybrid vectors are produced on the basis of E2a and/or E4 deleted rAd vectors in corresponding packaging cell lines. The maximal insert size in hybrid vectors is dictated by the packaging limit of first generation vectors (Ad.AAV) (<36kb) which are necessary intermediates for hybrid virus production at large scale. It is expected that stability and titer of Ad.AAV vectors with an 8kb globin gene cassette is comparable to the vector containing the 2.5-3.5kb cassette used in Ad.AAVBG, Ad.AAV1, and Ad.AAVSNori. The following example experiments address these issues.

Production of ΔAd.AAV^{fx} with large globin expression cassettes:

In order to improve the condition of sickle cell disease, the expression level of the transferred γ-lobin gene must be at least 50% of that of each endogenous βgene. These levels of transgene expression can only be achieved by using optimal expression cassettes, including extended LCRs and intron containing gamma genes. (Forrester, W.C., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 1359-1363; Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in*

Hematology, 35, 105-111; Martin, D. et al., 1996, *Current Opinion in Genetics and Development*, 6:488-95), So far, most of the γ-globin expression cassettes are designed for retroviral and rAAV vectors, thus, less than 5kb and have to be devoid of internal splice sites or poly adenylation signals. With integrating vectors described herein, it is possible to go beyond this size limitation. This allows one to improve γ -globin expression in bone marrow cells in terms of an adequate expression level and long term persistence. For this purpose, γ -globin constructs developed by Li et al (Emery, D. W., et al. 1999 *Hum Gene Ther* 10:877-88; Li, Q., et al. 1999. *Blood* 93:2208-16) or by Ellis et al (Ellis, J., et al., 1996, *EMBO J.*, 15, 562-568; Ellis, J., et al., 1997, *Nucleic Acids Res.* 25, 1296-1302) is chosen.

(i) The first cassette contains a γ -globin expression unit used in retroviral vectors. This allows for a direct comparison between the two systems. This construct includes the beta promoter from -127 to the beta initiation codon, which is connected in frame with the gamma coding region. This beta promoter is combined with the 300bp HS40 derived from the human alpha globin locus, which acts as a strong enhancer for globin expression. The globin gene is the 1.1kb version with intron 1 and partially deleted intron 2. A second cassette is generated containing the HS40 beta promoter and gamma globin gene with the complete 3.3kb gamma globin gene.

(ii) The second construct contains the 6.5kb beta μLCR, which confer a dominant chromatin opening activity and an adequate level of gamma globin expression in transgenic mice. The LCR is linked to the short 1.1kb version of the gamma globin gene or the complete 3.3kb gamma gene.

(iii) Additional globin expression cassette can be generated which include insulators, MARs or SARs, as well as other elements that can improve transgene expression from integrated vectors or in transgenic animals, like introns derived from the HPRT or hGH genes (Chung, J.H., et al., 1997, *Proc. Natl. Acad. Sci. USA* 94, 575-580; Dunaway, M., et al, 1993, *Mol. Cell. Biol.*, 17, 182-189; Felsenfeld, G., et al., 1996,

Proc. Natl. Acad. Sci. USA 93, 93840-9388; Klehr, D., et al., 1991, *Biochemistry*, 30, 1264-1270).

Transduction studies with ΔAd.AAVfx-globin vectors:

Transduction studies with globin-hybrid vectors are performed as described earlier (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13). Transduced CD34+ cells are submitted to differentiation in colony assays or analyzed *in vivo* expansion assays in SCID-NOD mice. MC-colonies or bone marrow cells from experimental mice are analyzed for globin expression. Gamma-globin expression is measured using fluorescent anti-gamma-globin antibodies. RNAase protection studies can be performed to specifically quantitate gamma globin mRNA in comparison with.-globin RNA. For these studies around 10^4-10^5 cells are needed per test.

Position effects:

In the absence of the LCR, globin genes are subjected to strong position effects when they are transferred into cultured CD34+ cells or erythroleukemic lines (Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111). Another concern is that site-specific integration of ΔAd.AAV/rep vectors into AAVS1 may silence transgene expression. If silencing happens, it can be overcome by incorporating LCRs such as the 6.5kb □ globin μLCR (Ellis, J., et al., 1996, *EMBO J.*, 15, 562-568; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111) or insulators into □Ad.AAV based expression units. Insulators are DNA elements that protect an integrated reporter gene from chromosomal position effects or that block enhancer activated transcription from a downward promoter. Insulator elements are known for Drosophila melanogaster genes (Gypsy, suppressor of Hairy wing, scs, scs', Fab-7). for the chicken beta-globin gene (HS4) and for the T cell receptor (BEAD1; 14, 21.25). Specifically, the Drosophila gypsy or the beta globin insulator can be inserted as two copies flanking the globin expression cassette into hybrid vectors. The position effects can be examined in transduced MC-colonies based on the

analysis of integrated vector DNA (see Fig. 29) and gamma-globin mRNA quantification. Analogous studies can be performed on transduced human bone marrow cells obtained after transplantation of infected CD34+ cells into SCID-NOD mice.

Intron effects on gamma-globin expression:

A number of reports reveal that the deletion of globin introns, particularly the second intron of the beta and gamma genes, decrease globin mRNA stability and thus the expression level (Antoniou, M. et al., 1998, *Nucleic Acid Res.*, 26:721-9). RNA viruses such as onco-retro, lenti- and foami viruses are problematic as vehicles for intron-containing transgenes. Because ΔAd.AAV is a DNA virus, it should package globin introns and LCRs if necessary without the decreased titers and rearrangements observed with retroviral vectors.

APPENDIX I

HUMAN AND ANIMAL ADENOVIRUSES AVAILABLE FROM AMERICAN TYPE CULTURE COLLECTION

1: Adenovirus Type 21 ATCC VR-1099 (NIAID V-221-002-014)
2: SA18 (Simian adenovirus 18) ATCC VR-943 Classification
3: SA17 (Simian adenovirus 17) ATCC VR-942 Classification
4: Adenovirus Type 47 ATCC VR-1309 Classification: Adenov
5: Adenovirus Type 44 ATCC VR-1306 Classification: Adenov
6: Avian adenovirus Type 4 ATCC VR-829 Classification: Ad
7: Avian adenovirus Type 5 ATCC VR-830 Classification: Ad
8: Avian adenovirus Type 7 ATCC VR-832 Classification: Ad
9: Avian adenovirus Type 8 ATCC VR-833 Classification: Ad
10: Avian adenovirus Type 9 ATCC VR-834 Classification: Ad
11: Avian adenovirus Type 10 ATCC VR-835 Classification: A
12: Avian adenovirus Type 2 ATCC VR-827 Classification: Ad
13: Adenovirus Type 45 ATCC VR-1307 Classification: Adenov
14: Adenovirus Type 38 ATCC VR-988 Permit: PHS permit requ
15: Adenovirus Type 46 ATCC VR-1308 Classification: Adenov
16: Simian adenovirus ATCC VR-541 Classification: Adenovir
17: SA7 (Simian adenovirus 16) ATCC VR-941 Classification:
18: Frog adenovirus (FAV-1) ATCC VR-896 Classification: Ad
19: Adenovirus Type 48 (candidate) ATCC VR-1406 Classifica
20: Adenovirus Type 42 ATCC VR-1304 Classification: Adenov

21: <u>Adenovirus type 49 (candidate) ATCC VR-1407 Classifica</u>

22: <u>Adenovirus Type 43 ATCC VR-1305 Classification: Adenov</u>

23: <u>Avian adenovirus Type 6 ATCC VR-831 Permit: USDA permi</u>

24: <u>Avian adenovirus Type 3 (Inclusion body hepatitis virus)</u>

25: <u>Bovine adenovirus Type 3 ATCC VR-639 Classification: A</u>

26: <u>Bovine adenovirus Type 6 ATCC VR-642 Permit: USDA perm</u>

27: <u>Canine adenovirus ATCC VR-800 Classification: Adenovir</u>

28: <u>Bovine adenovirus Type 5 ATCC VR-641 Permit: USDA perm</u>

29: <u>Adenovirus Type 36 ATCC VR-913 Classification: Adenovi</u>

30: <u>Ovine adenovirus type 5 ATCC VR-1343 Classification: A</u>

31: <u>Adenovirus Type 29 ATCC VR-272 Classification: Adenovi</u>

32: <u>Swine adenovirus ATCC VR-359 Classification: Adenoviru</u>

33: <u>Bovine adenovirus Type 4 ATCC VR-640 Permit: USDA perm</u>

34: <u>Bovine adenovirus Type 8 ATCC VR-769 Permit: USDA perm</u>

35: <u>Bovine adenovirus Type 7 ATCC VR-768 Permit: USDA perm</u>

36: <u>Adeno-associated virus Type 2 (AAV-2H) ATCC VR-680 Cla</u>

37: <u>Adenovirus Type 4 ATCC VR-4 Classification: Adenovirus</u>

38: <u>Adeno-associated virus Type 3 (AAV-3H) ATCC VR-681 Cla</u>

39: <u>Peromyscus adenovirus ATCC VR-528 Classification: Aden</u>

40: <u>Adenovirus Type 15 ATCC VR-661 Classification: Adenovi</u>

41: <u>Adenovirus Type 20 ATCC VR-662 Classification: Adenovi</u>

42: <u>Chimpanzee adenovirus ATCC VR-593 Classification: Aden</u>

43: <u>Adenovirus Type 31 ATCC VR-357 Classification: Adenovi</u>

44: <u>Adenovirus Type 25 ATCC VR-223 Classification: Adenovi</u>

45: Chimpanzee adenovirus ATCC VR-592 Classification: Aden
46: Chimpanzee adenovirus ATCC VR-591 Classification: Aden
47: Adenovirus Type 26 ATCC VR-224 Classification: Adenovi
48: Adenovirus Type 19 ATCC VR-254 Classification: Adenovi
49: Adenovirus Type 23 ATCC VR-258 Classification: Adenovi
50: Adenovirus Type 28 ATCC VR-226 Classification: Adenovi
51: Adenovirus Type 6 ATCC VR-6 Classification: Adenovirus
52: Adenovirus Type 2 Antiserum: ATCC VR-1079 AS/Rab (NIA
53: Adenovirus Type 6 ATCC VR-1083 (NIAID V-206-003-014)
54: Ovine adenovirus type 6 ATCC VR-1340 Classification: A
55: Adenovirus Type 3 ATCC VR-847 (Derived from NIAID V-20)
56: Adenovirus Type 7 ATCC VR-7 Classification: Adenovirus
57: Adenovirus Type 39 ATCC VR-932 Classification: Adenovi
58: Adenovirus Type 3 ATCC VR-3 Classification: Adenovirus
59: Bovine adenovirus Type 1 ATCC VR-313 Classification: A
60: Adenovirus Type 14 ATCC VR-15 Classification: Adenovir
61: Adenovirus Type 1 ATCC VR-1078 (NIAID V-201-001-014)
62: Adenovirus Type 21 ATCC VR-256 Classification: Adenovi
63: Adenovirus Type 18 ATCC VR-1095 (NIAID V-218-003-014)
64: Baboon adenovirus ATCC VR-275 Classification: Adenovir
65: Adenovirus Type 10 ATCC VR-11 Classification: Adenovir
66: Adenovirus Type 33 ATCC VR-626 Classification: Adenovi

67: <u>Adenovirus Type 34 ATCC VR-716 Classification: Adenovi</u>

68: <u>Adenovirus Type 15 ATCC VR-16 Classification: Adenovir</u>

69: <u>Adenovirus Type 22 ATCC VR-257 Classification: Adenovi</u>

70: <u>Adenovirus Type 24 ATCC VR-259 Classification: Adenovi</u>

71: <u>Adenovirus Type 17 ATCC VR-1094 (NIAID V-217-003-014)</u>

72: <u>Adenovirus Type 4 ATCC VR-1081 (NIAID V-204-003-014)</u>

73: <u>Adenovirus Type 16 ATCC VR-17 Classification: Adenovir</u>

74: <u>Adenovirus Type 17 ATCC VR-18 Classification: Adenovir</u>

75: <u>Adenovirus Type 16 ATCC VR-1093 (NIAID V-216-003-014)</u>

76: <u>Bovine adenovirus Type 2 ATCC VR-314 Classification: A</u>

77: <u>SV-30 ATCC VR-203 Classification: Adenovirus, Simian (</u>

78: <u>Adenovirus Type 32 ATCC VR-625 Classification: Adenovi</u>

79: <u>Adenovirus Type 20 ATCC VR-255 Classification: Adenovi</u>

80: <u>Adenovirus Type 13 ATCC VR-14 Classification: Adenovir</u>

81: <u>Adenovirus Type 14 ATCC VR-1091 (NIAID V-214-001-014)</u>

82: <u>Adenovirus Type 18 ATCC VR-19 Classification: Adenovir</u>

83: <u>SV-39 ATCC VR-353 Classification: Adenovirus, Simian (</u>

84: <u>Adenovirus Type 11 ATCC VR-849 (Derived from NIAID V-2</u>

85: <u>Duck adenovirus (Egg drop syndrome) ATCC VR-921 Permi</u>

86: <u>Adenovirus Type 1 ATCC VR-1 Classification: Adenovirus</u>

87: <u>Chimpanzee adenovirus ATCC VR-594 Classification: Aden</u>

88: <u>Adenovirus Type 15 ATCC VR-1092 (NIAID V-215-003-014)</u>

89: <u>Adenovirus Type 13 ATCC VR-1090 (NIAID V-213-003-014)</u>
90: <u>Adenovirus Type 8 ATCC VR-1368 (Derived from NIAID V-20</u>
91: <u>SV-31 ATCC VR-204 Classification: Adenovirus, Simian (</u>
92: <u>Adenovirus Type 9 ATCC VR-1086 (NIAID V-209-003-014)</u>
93: <u>Mouse adenovirus ATCC VR-550 Classification: Adenoviru</u>
94: <u>Adenovirus Type 9 ATCC VR-10 Classification: Adenoviru</u>
95: <u>Adenovirus Type 41 ATCC VR-930 Classification: Adenovi</u>
96: <u>CI ATCC VR-20 Classification: Adenovirus, Simian (Mast</u>
97: <u>Adenovirus Type 40 ATCC VR-931 Classification: Adenovi</u>
98: <u>Adenovirus Type 37 ATCC VR-929 Classification: Adenovi</u>
99: <u>Marble spleen disease virus (Hemorrhagic enteritis virus</u>
100: <u>Adenovirus Type 35 ATCC VR-718 Classification: Adenovi</u>
101: <u>SV-32 (M3) ATCC VR-205 Classification: Adenovirus, Sim</u>
102: <u>Adenovirus Type 28 ATCC VR-1106 (NIAID V-228-003-014)</u>
103: <u>Adenovirus Type 10 ATCC VR-1087 (NIAID V-210-003-014)</u>
104: <u>Adenovirus Type 20 ATCC VR-1097 (NIAID V-220-003-014)</u>
105: <u>Adenovirus Type 21 ATCC VR-1098 (NIAID V-221-011-014)</u>
106: <u>Adenovirus Type 25 ATCC VR-1103 (NIAID V-225-003-014)</u>
107: <u>Adenovirus Type 26 ATCC VR-1104 (NIAID V-226-003-014)</u>
108: <u>Adenovirus Type 31 ATCC VR-1109 (NIAID V-231-001-014)</u>
109: <u>Adenovirus Type 19 ATCC VR-1096 (NIAID V-219-002-014)</u>
110: <u>SV-36 ATCC VR-208 Classification: Adenovirus, Simian (</u>

111: <u>SV-38 ATCC VR-355 Classification: Adenovirus, Simian (</u>
112: <u>SV-25 (M8) ATCC VR-201 Classification: Adenovirus, Sim</u>
113: <u>SV-15 (M4) ATCC VR-197 Classification: Adenovirus, Sim</u>
114: <u>Adenovirus Type 22 ATCC VR-1100 (NIAID V-222-003-014)</u>
115: <u>SV-23 (M2) ATCC VR-200 Classification: Adenovirus, Sim</u>
116: <u>Adenovirus Type 11 ATCC VR-12 Classification: Adenovir</u>
117: <u>Adenovirus Type 24 ATCC VR-1102 (NIAID V-224-003-014)</u>
118: <u>Avian adenovirus Type 1 (Chicken Embryo Lethal Orphan: C</u>
119: <u>SV-11 (M5) ATCC VR-196 Classification: Adenovirus, Sim</u>
120: <u>Adenovirus Type 5 ATCC VR-5 Classification: Adenovirus</u>
121: <u>Adenovirus Type 23 ATCC VR-1101 (NIAID V-223-003-014)</u>
122: <u>SV-27 (M9) ATCC VR-202 Classification: Adenovirus, Sim</u>
123: <u>Avian adenovirus Type 2 (GAL) ATCC V-280 Classificati</u>
124: <u>SV-1 (M1) ATCC VR-195 Classification: Adenovirus, Simi</u>
125: <u>SV-17 (M6) ATCC VR-198 Classification: Adenovirus, Sim</u>
126: <u>Adenovirus Type 29 ATCC VR-1107 (NIAID V-229-003-014)</u>
127: <u>Adenovirus Type 2 ATCC VR-846 Classification: Adenovir</u>
128: <u>SV-34 ATCC VR-207 Classification: Adenovirus, Simian (</u>
129: <u>SV-20 (M7) ATCC VR-199 Classification: Adenovirus, Sim</u>
130: <u>SV-37 ATCC VR-209 Classification: Adenovirus, Simian (</u>
131: <u>SV-33 (M10) ATCC VR-206 Classification: Adenovirus, Si</u>

132: Avian adeno-associated virus ATCC VR-865 Classificatio

133: Adeno-associated (satellite) virus Type 4 ATCC VR-646

134: Adenovirus Type 30 ATCC VR-273 Classification: Adenovi

135: Adeno-associated (satellite) virus Type 1 ATCC VR-645

136: Infectious canine hepatitis (Rubarth's disease, Fox ence

137: Adenovirus Type 27 ATCC VR-1105 (NIAID V-227-003-014)

138: Adenovirus Type 12 ATCC VR-863 (Derived from NIAID V-2

139: Adeno-associated virus Type 2 (molecularly cloned) ATCC

140: Adenovirus Type 7a ATCC VR-848 (Derived from NIAID V-2

What is claimed:

1. A first generation recombinant adenovirus vector a portion of which integrates into a host cell genome, comprising:
   a) A left adenovirus inverted terminal repeat sequence;
   b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
   c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
   d) At least one adenoviral sequence which directs adenoviral replication; and
   e) A right adenoviral inverted terminal repeat sequence,

   wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

2. The adenovirus vector of claim 1, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype.

3. The adenovirus vector of claim 1, wherein the sequence which directs adenoviral replication comprises a sequence on the anti-parallel strand which encodes an adenoviral fiber protein including a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

4. The adenovirus vector of claim 3, wherein the sequence on the anti-sense strand which encodes the fiber tail is from the same serotype as the adenoviral inverted repeat sequence.

5. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:
   a) A left cassette inverted terminal repeat sequence;
   b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the polyadenylation sequence;

d) A promoter sequence 3' to the polyadenylation sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

6. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;

b) A promoter sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the promoter sequence;

d) A polyadenylation sequence 3' to the transgene sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

7. The adenovirus vector of claim 5 or 6, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

8. The adenovirus vector of claim 5 or 6, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

9. The adenovirus vector of claim 8, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

10. The adenovirus vector of claim 8, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

11. The adenovirus vector of claim 8, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

12. The transgene cassette of claim 5 or 6 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

13. The transgene cassette of claim 5 or 6, further comprising an insulator element.

14. The transgene cassette of claim 5 or 6, further comprising a bacterial origin of replication.

15. The adenoviral vector of claim 1, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

16. A first generation recombinant adenovirus vector which targets a host cell of interest and a portion of which integrates into the host cell genome so targeted, comprising two DNA strands, each strand being antiparallel to the other, the first strand comprising:

a) A left adenovirus inverted terminal repeat sequence;
b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
d) At least one adenoviral sequence which directs adenoviral replication; and
e) A right adenoviral inverted terminal repeat sequence,

wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome, and wherein the second strand comprises a sequence which encodes an adenoviral fiber protein that permits targeting of the vector into the host cell of interest.

17. The adenovirus vector of claim 16, wherein the adenoviral protein includes a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

18. The adenovirus vector of claim 16, wherein the left and right adenovirus inverted terminal repeat sequences and the packaging sequence are from the same adenoviral serotype.

19. The adenovirus vector of claim 17, wherein the fiber tail is from the same serotype as the left and right adenoviral inverted repeat sequences.

20. The adenovirus vector of claim 17, wherein the fiber shaft is from a different serotype as the left and right adenoviral inverted repeat sequences.

21. The adenovirus vector of claim 20, wherein the fiber shaft is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

22. The adenovirus vector of claim 17, wherein the fiber shaft comprises a shortened length.

23. The adenovirus vector of claim 17, wherein the fiber knob is from a different serotype as the left and right adenoviral inverted repeat sequences.

24. The adenovirus vector of claim 23, wherein the fiber knob is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

25. The adenovirus vector of claim 17, wherein the fiber knob is a modified fiber knob protein comprising the G-H loop replaced with a heterologous peptide ligand sequence which binds to at least one surface protein on the host cell of interest.

26. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:
    a) A left cassette inverted terminal repeat sequence;
    b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;
    c) A transgene sequence 3' to the polyadenylation sequence;
    d) A promoter sequence 3' to the polyadenylation sequence; and
    e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

27. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:
    a) A left cassette inverted terminal repeat sequence;
    b) A promoter sequence 3' to the left inverted terminal repeat sequence;
    c) A transgene sequence 3' to the promoter sequence;
    d) A polyadenylation sequence 3' to the transgene sequence; and
    e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

28. The adenovirus vector of claim 26 or 27, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

29. The adenovirus vector of claim 26 or 27, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

30. The adenovirus vector of claim 24, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

31. The adenovirus vector of claim 24, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

32. The adenovirus vector of claim 24, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

33. The transgene cassette of claim 26 or 27 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

34. The transgene cassette of claim 26 or 27, further comprising an insulator element.

35. The transgene cassette of claim 26 or 27, further comprising a bacterial origin of replication.

36. The adenoviral vector of claim 16, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

37. A recombinant gutless adenovirus vector a portion of which integrates into a host cell genome, comprising:

a) A left adenovirus inverted terminal repeat sequence;
b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
c) A transgene cassette sequence 3' to the adenoviral packaging sequence; and
d) A right adenoviral inverted terminal repeat sequence,

wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

38. The adenovirus vector of claim 36, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype. *(this is the base vector)*

39. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:
 a) A left cassette inverted terminal repeat sequence;
 b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;
 c) A transgene sequence 3' to the polyadenylation sequence;
 d) A promoter sequence 3' to the polyadenylation sequence; and
 e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

40. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:
 b) A left cassette inverted terminal repeat sequence;
 c) A promoter sequence 3' to the left inverted terminal repeat sequence;
 d) A transgene sequence 3' to the promoter sequence;
 e) A polyadenylation sequence 3' to the transgene sequence; and
 f) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

41. The adenovirus vector of claim 38 or 39, wherein the left and the right inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

42. The adenovirus vector of claim 38 or 39, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

43. The adenovirus vector of claim 41, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

44. The adenovirus vector of claim 41, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentomycin.

45. The adenovirus vector of claim 41, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

46. The adenovirus vector of claim 38 or 39 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

47. The adenovirus vector of claim 38 or 39, further comprising an insulator element.

48. The adenovirus vector of claim 38 or 39, further comprising a bacterial origin of replication.

49. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 5' portion of a gene of interest.

50. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 3' portion of a gene of interest.

51. A method of producing a resolved gutless adenovirus vector in a suitable cell, said method comprising introducing a first and a second adenovirus vectors of claim 1 or 16 into the cell under suitable conditions so that the recombinant adenovirus vectors undergo homologous recombination thereby producing a resolved gutless adenovirus vector.

52. A resolved gutless adenovirus vector produced by the method of claim 50.

53. The method of claim 50, wherein the first adenovirus vector comprises a transgene cassette having a 5' portion of a gene of interest, and wherein the second adenovirus vector comprises a transgene cassette having a 3' portion of the gene of interest, and wherein a part of the 5' portion overlaps with a part of the 3' portion so that homologous recombination occurs.

54. A method of producing a resolved gutless recombinant Ad vector by homologous recombination in a suitable cell, said method comprising contacting two parental recombinant Ad vectors, each comprising a transgene cassette containing a portion of a selected transgene with a region of overlapping homology, so that the first and second parental recombinant Ad vectors undergo homologous recombination at the region of overlapping homology, resulting in a resolved recombinant gutless Ad vector having both portions of the selected transgene, and wherein the selected transgene is within a transgene cassette flanked by a pair of ITRs.

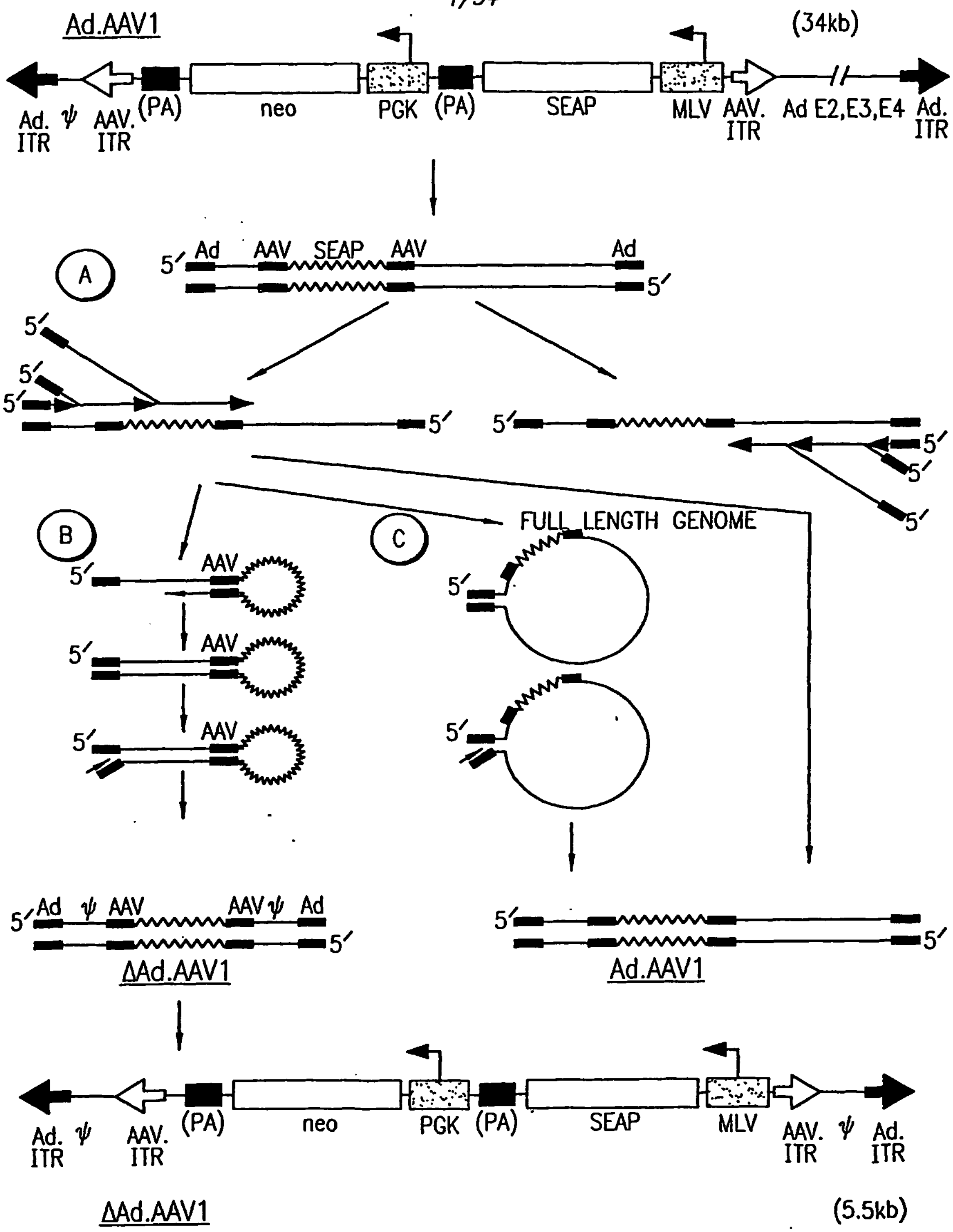
55. A resolved gutless adenovirus vector produced by the method of claim 53.

56. An adenovirus library comprising a plurality of adenovirus vectors expressing fiber proteins which are displayed and modified by random peptide insertions.

57. The library of claim 55, wherein said fiber protein so displayed comprises a random peptide substituted in the G-H loop of the fiber protein knob domain.

58. A screening method for targeting adenovirus vectors for gene therapy comprising contacting the adenovirus library of claim 55 with a plurality of cells so that the cells are transduced with the adenovirus vectors of the adenovirus library transduction occurs and detecting the cells so transduced.

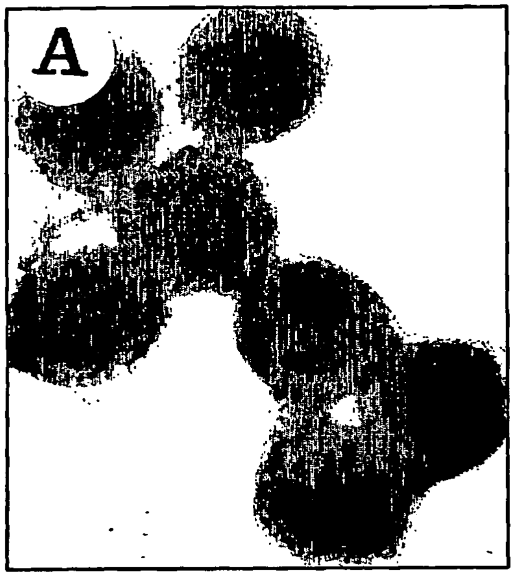
59. The adenovirus vector of claim 5, 6, 26, 27, 38, or 39 further comprising a nucleotide sequence encoding a rep78 protein.



Ad.AAV1
(34kb)
Ad. ITR
ψ
AAV. ITR
(PA)
neo
PGK
(PA)
SEAP
MLV
AAV. ITR
Ad E2,E3,E4
Ad. ITR
A
5′
Ad
AAV
SEAP
AAV
Ad
5′
B
AAV
C
FULL LENGTH GENOME
5′
Ad
ψ
AAV
AAV
ψ
Ad
5′
ΔAd.AAV1
Ad.AAV1
Ad. ITR
ψ
AAV. ITR
(PA)
neo
PGK
(PA)
SEAP
MLV
AAV. ITR
ψ
Ad. ITR
ΔAd.AAV1
(5.5kb)

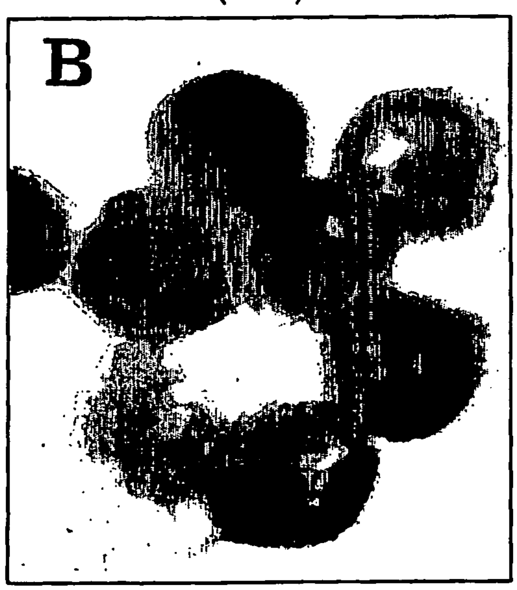
FIG. 1

Ad.AAV
(33kb)



50nm

ΔAd.AAV
(5.5kb)



50nm

FIG. 2

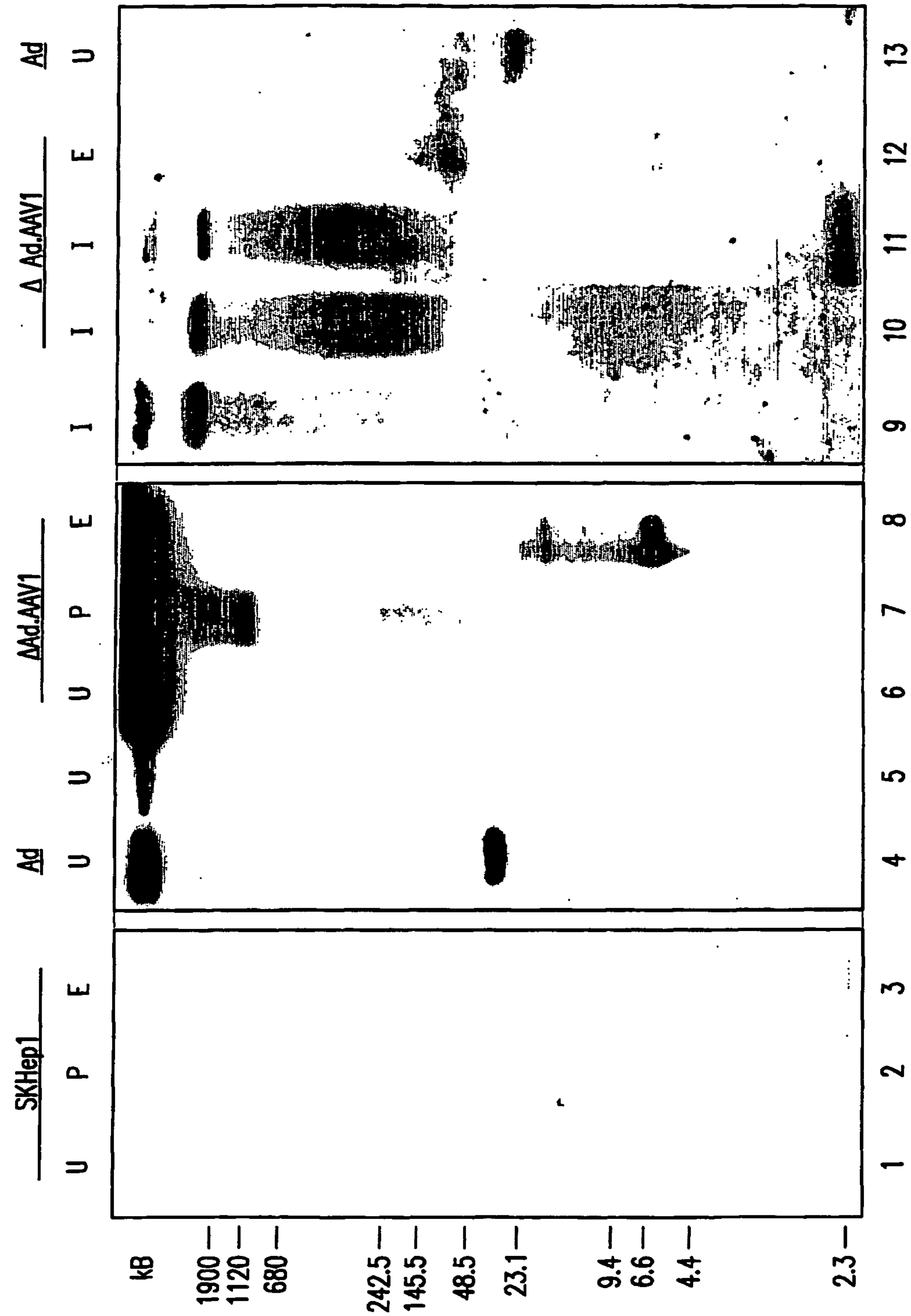

SKHep1
U P E
1 2 3
Ad
U
4
ΔAd.AAV1
U U P E
5 6 7 8
Δ Ad.AAV1
I I I E
9 10 11 12
Ad
U
13
kB
1900
1120
680
242.5
145.5
48.5
23.1
9.4
6.6
4.4
2.3

FIG. 3

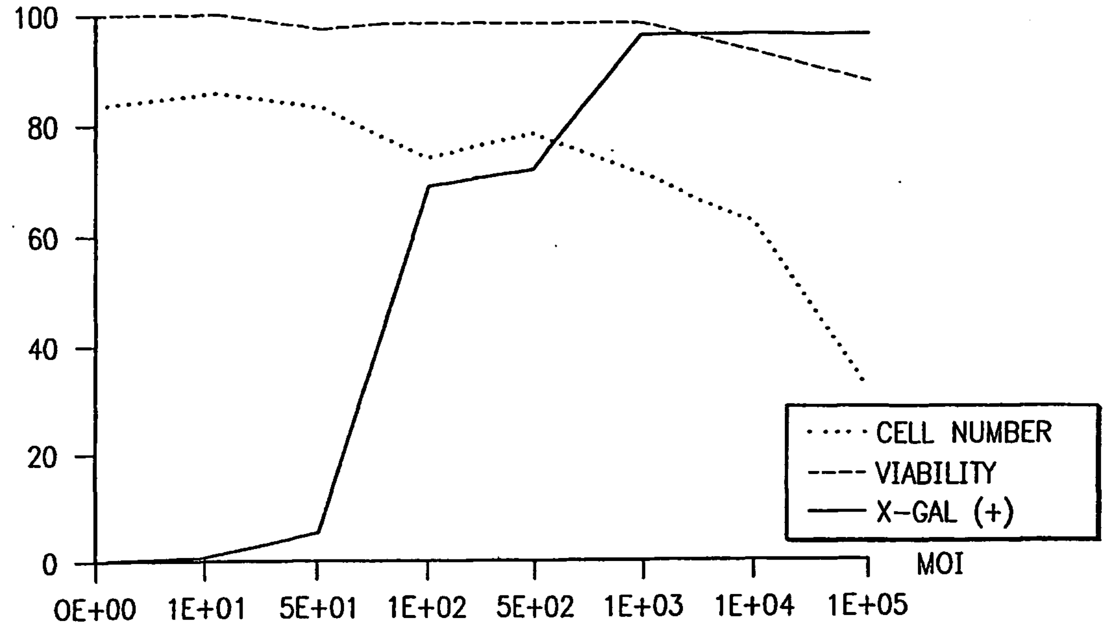


FIG. 4A K562

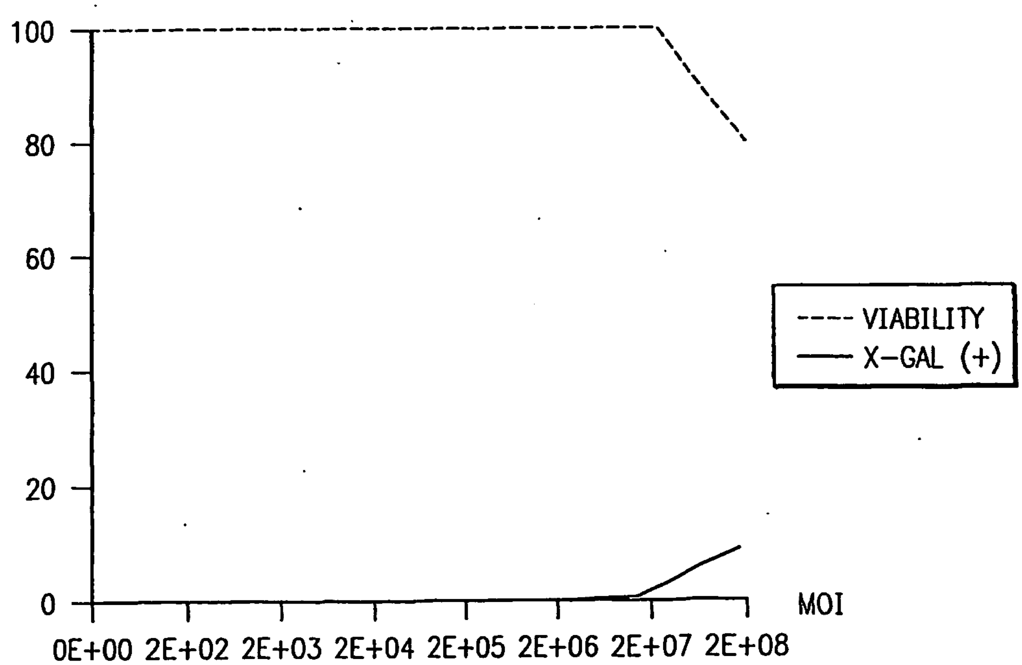


FIG. 4B CD34+

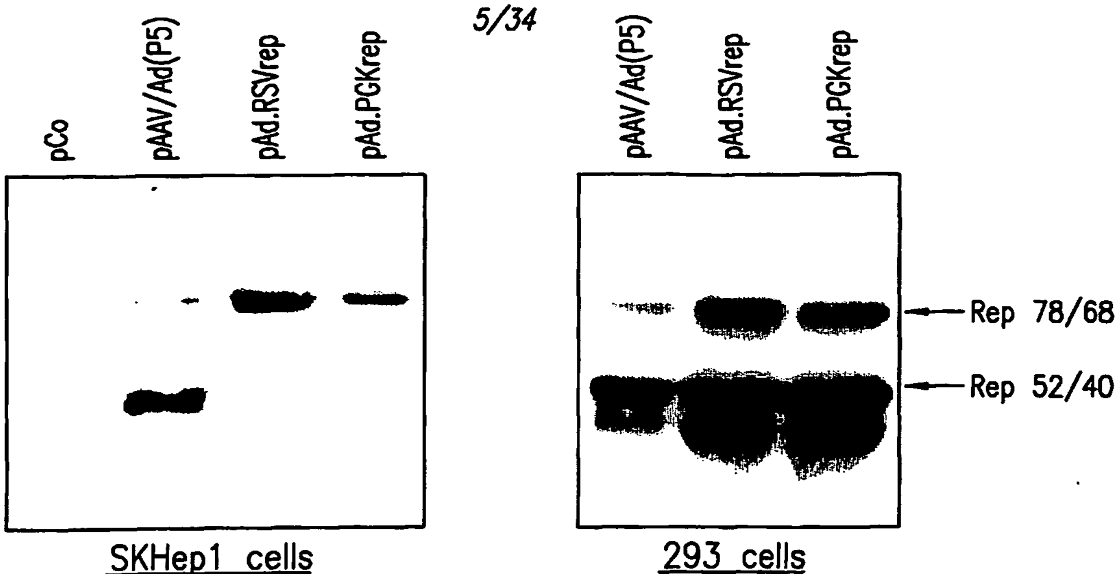

pCo
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
Rep 78/68
Rep 52/40
SKHep1 cells
293 cells

FIG. 5

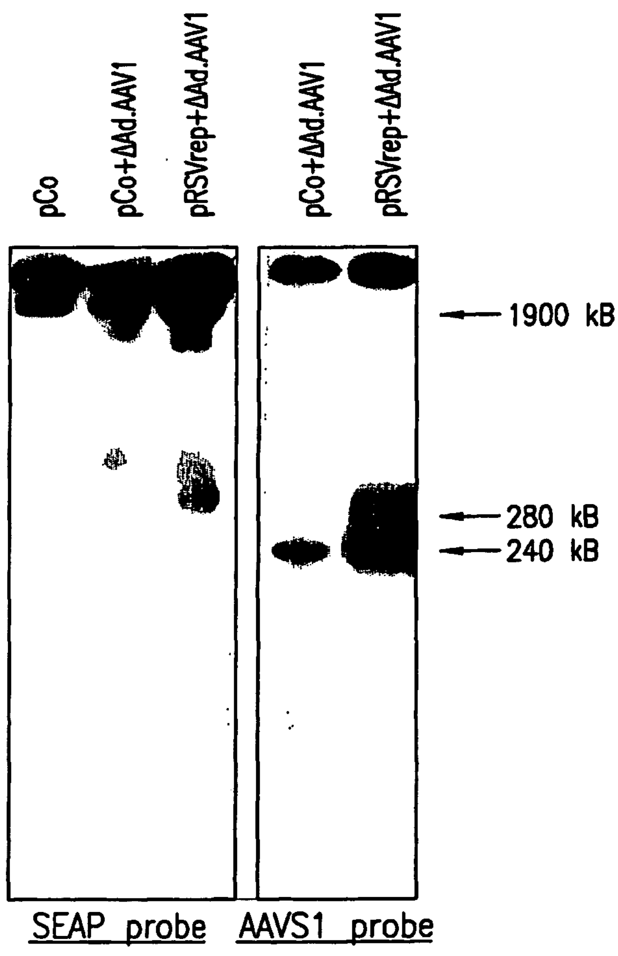

pCo
pCo+ΔAd.AAV1
pRSVrep+ΔAd.AAV1
pCo+ΔAd.AAV1
pRSVrep+ΔAd.AAV1
1900 kB
280 kB
240 kB
SEAP probe
AAVS1 probe

FIG. 6

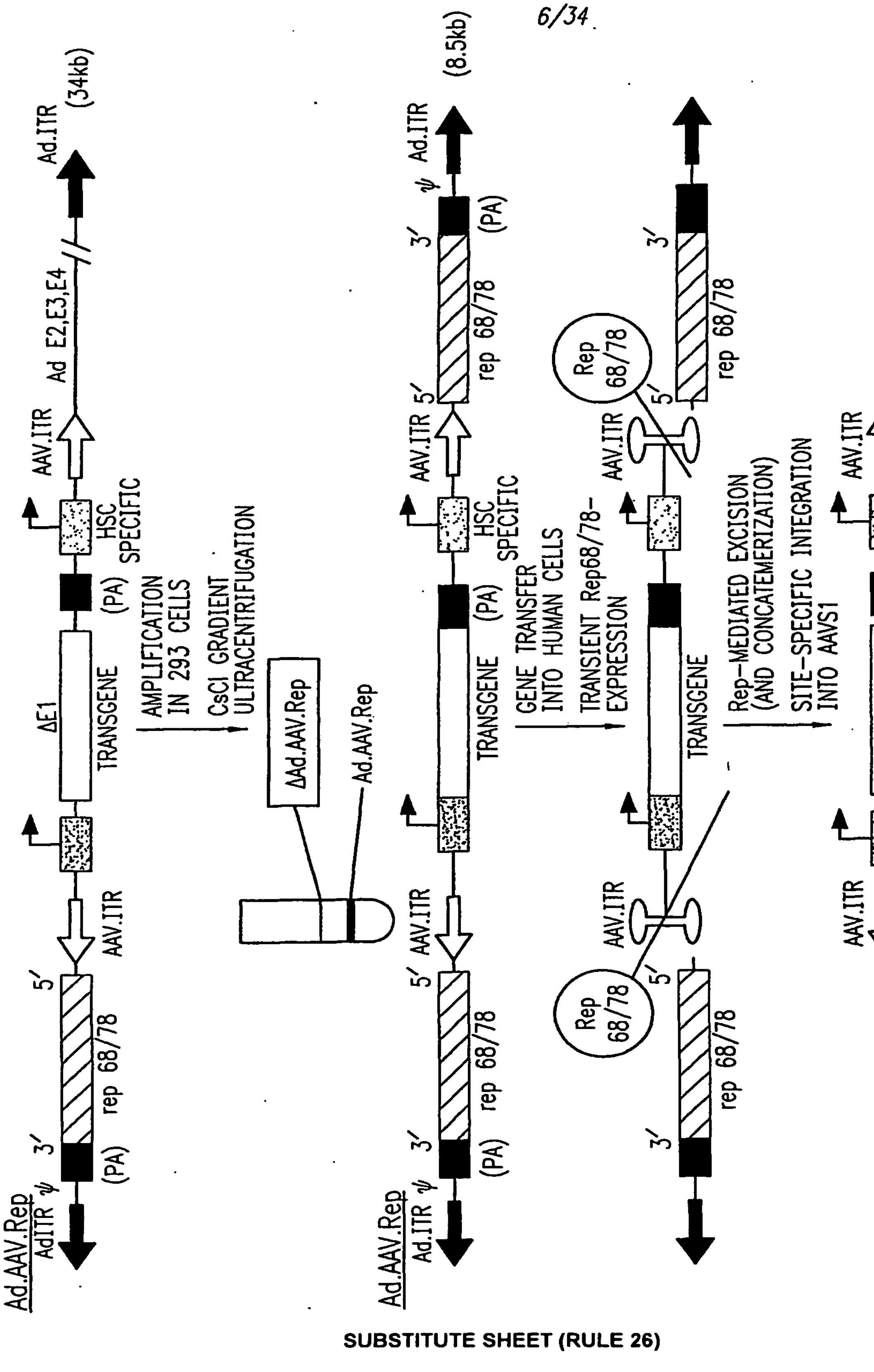

Ad.AAV.Rep
AdITR ψ
(PA)
3′
rep 68/78
5′
AAV.ITR
ΔE1
TRANSGENE
(PA)
HSC SPECIFIC
AAV.ITR
Ad E2,E3,E4
Ad.ITR
(34kb)
AMPLIFICATION IN 293 CELLS
CsCl GRADIENT ULTRACENTRIFUGATION
ΔAd.AAV.Rep
Ad.AAV.Rep
Ad.AAV.Rep
Ad.ITR ψ
(8.5kb)
GENE TRANSFER INTO HUMAN CELLS
TRANSIENT Rep68/78-EXPRESSION
Rep 68/78
Rep 68/78
Rep-MEDIATED EXCISION (AND CONCATEMERIZATION)
SITE-SPECIFIC INTEGRATION INTO AAVS1
CHROMOSOME 19

FIG. 7

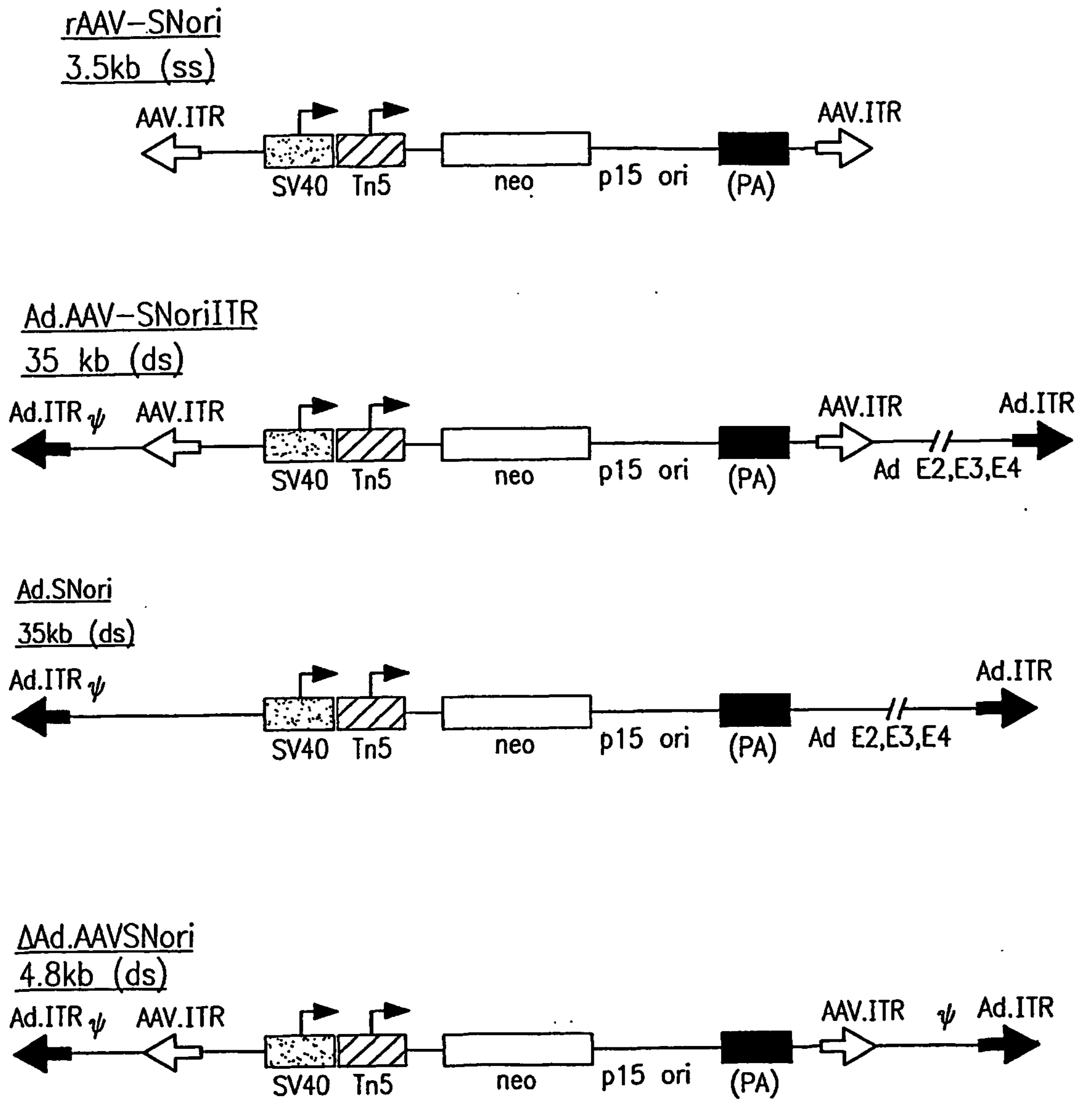

rAAV-SNori
3.5kb (ss)
AAV.ITR
AAV.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad.AAV-SNoriITR
35 kb (ds)
Ad.ITR
AAV.ITR
AAV.ITR
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad E2,E3,E4
Ad.SNori
35kb (ds)
Ad.ITR
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad E2,E3,E4
ΔAd.AAVSNori
4.8kb (ds)
Ad.ITR
AAV.ITR
AAV.ITR
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)

FIG. 8A

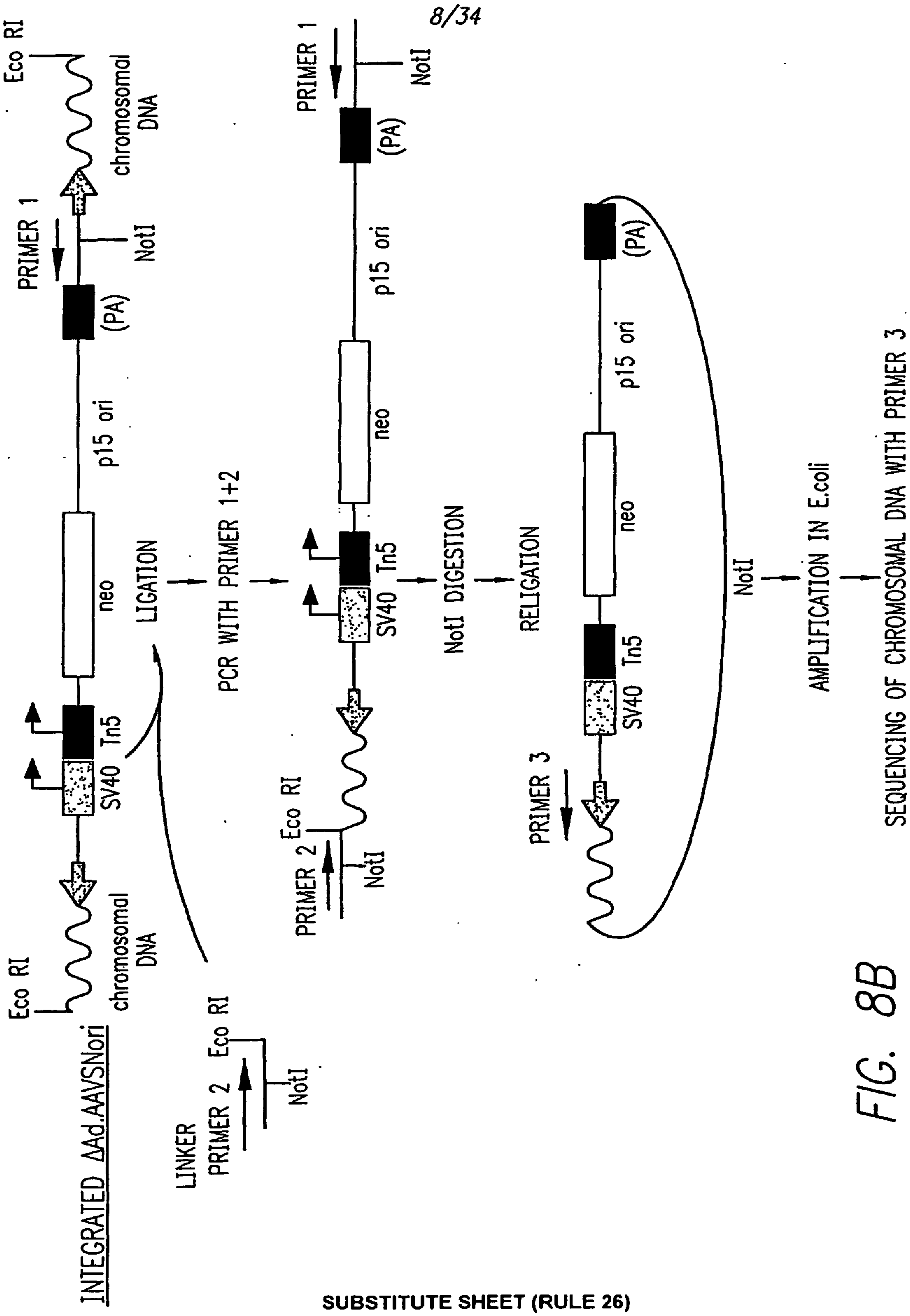

INTEGRATED ΔAd.AAVSNori
Eco RI
chromosomal DNA
SV40
Tn5
neo
p15 ori
(PA)
PRIMER 1
NotI
Eco RI
chromosomal DNA
LINKER
PRIMER 2
Eco RI
NotI
LIGATION
PCR WITH PRIMER 1+2
PRIMER 2
Eco RI
NotI
SV40
Tn5
neo
p15 ori
(PA)
PRIMER 1
NotI
NotI DIGESTION
RELIGATION
PRIMER 3
SV40
Tn5
neo
p15 ori
(PA)
NotI
AMPLIFICATION IN E.coli
SEQUENCING OF CHROMOSOMAL DNA WITH PRIMER 3

FIG. 8B

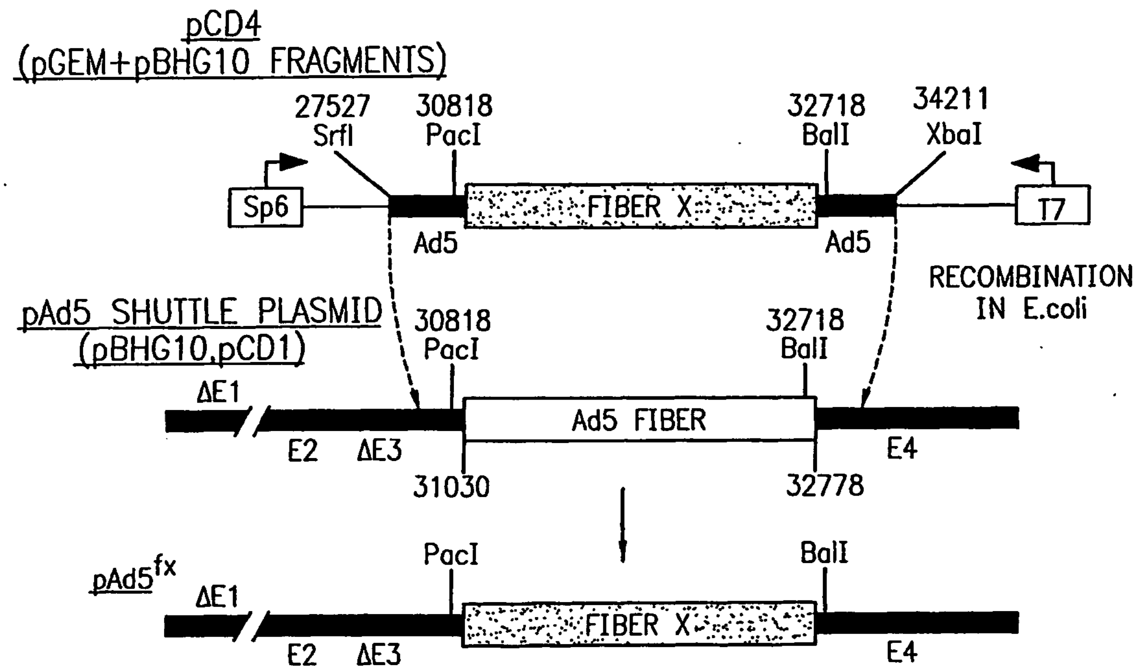

pCD4
(pGEM+pBHG10 FRAGMENTS)
27527
SrfI
30818
PacI
32718
BalI
34211
XbaI
Sp6
FIBER X
T7
Ad5
Ad5
RECOMBINATION
IN E.coli
pAd5 SHUTTLE PLASMID
(pBHG10,pCD1)
30818
PacI
32718
BalI
ΔE1
Ad5 FIBER
E2
ΔE3
E4
31030
32778
pAd5fx
PacI
BalI
ΔE1
FIBER X
E2
ΔE3
E4

FIG. 9

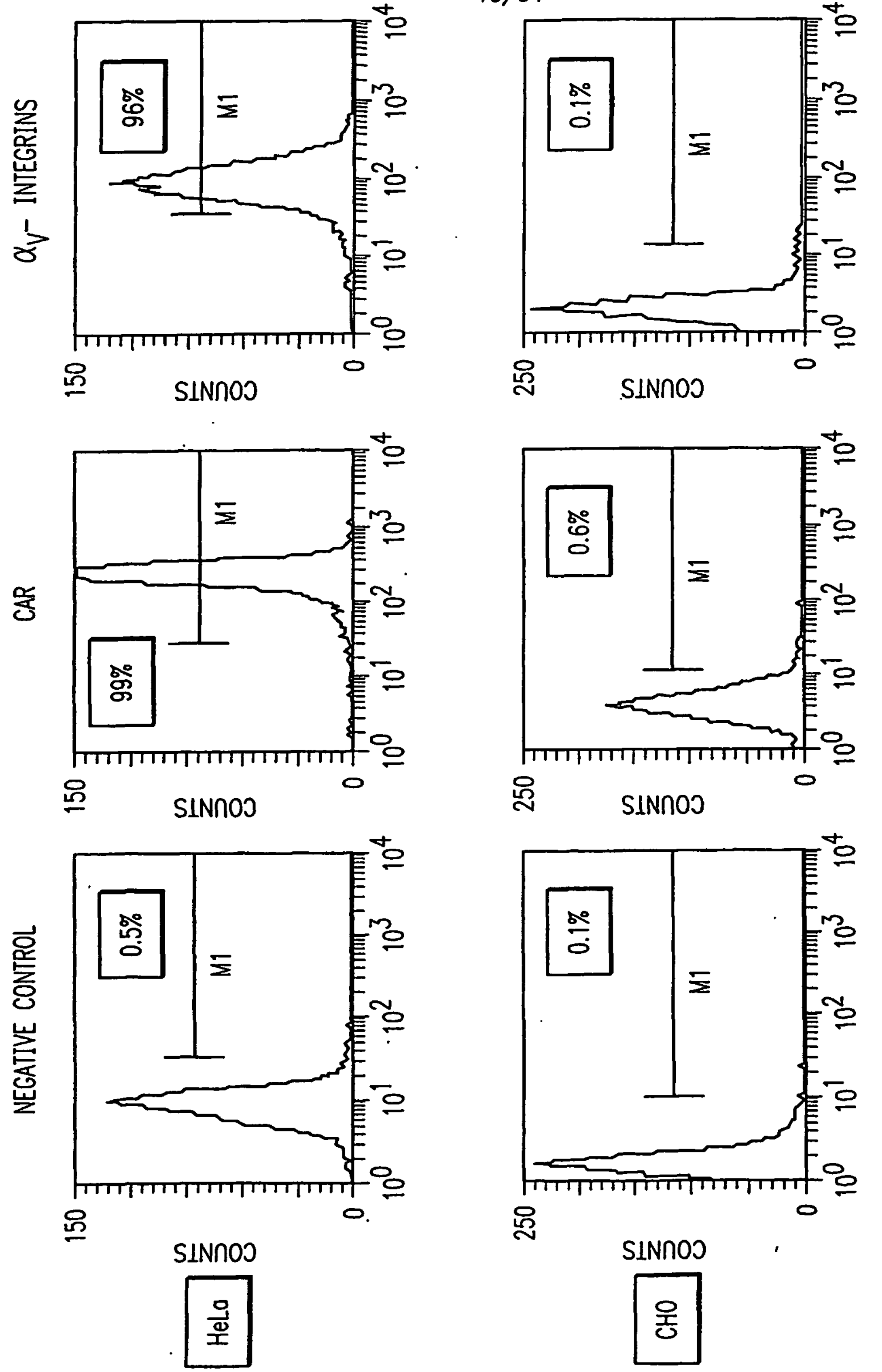

NEGATIVE CONTROL
CAR
α_V- INTEGRINS
HeLa
CHO
COUNTS
M1
0.5%
99%
96%
0.1%
0.6%
0.1%

FIG. 10–i

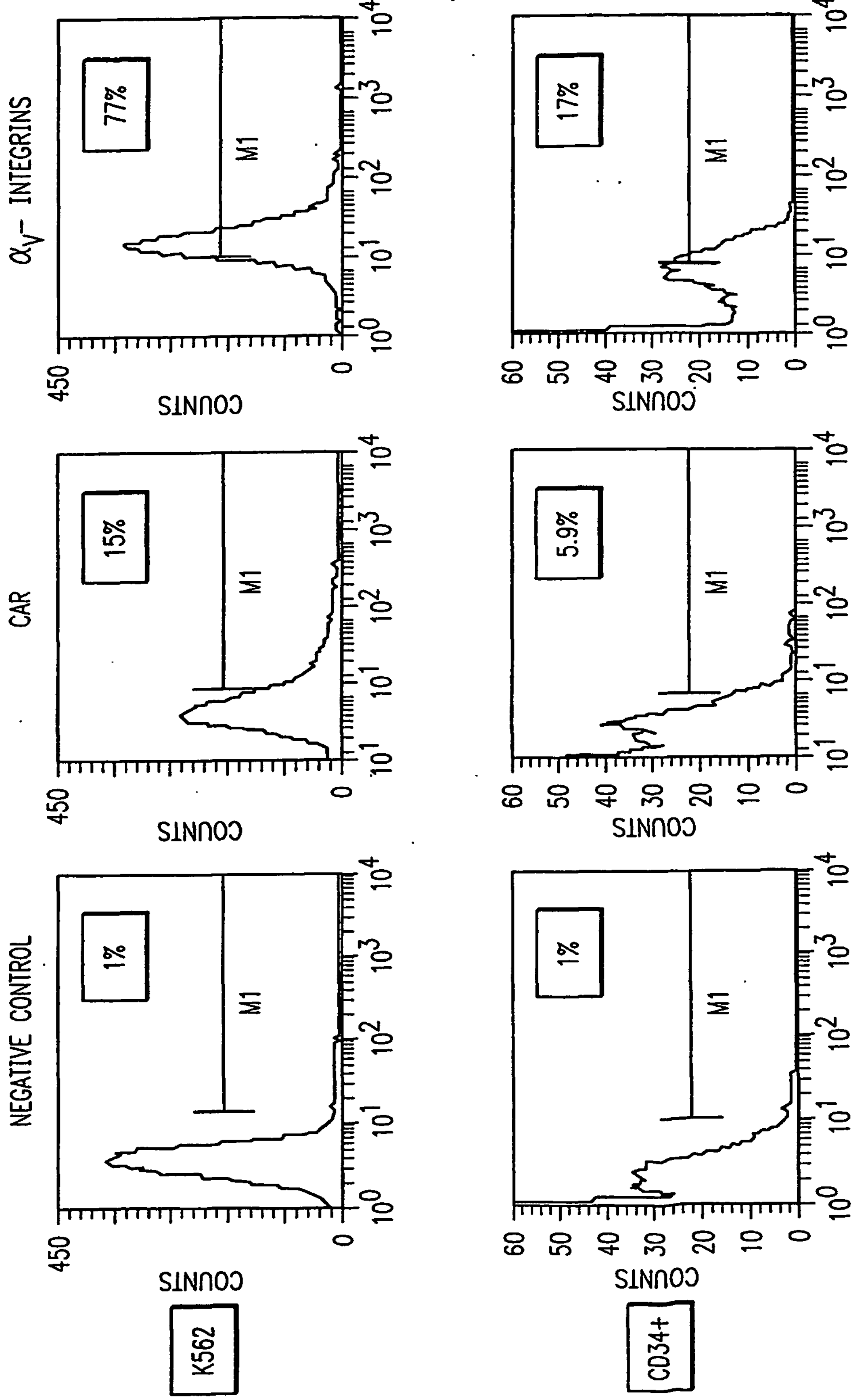
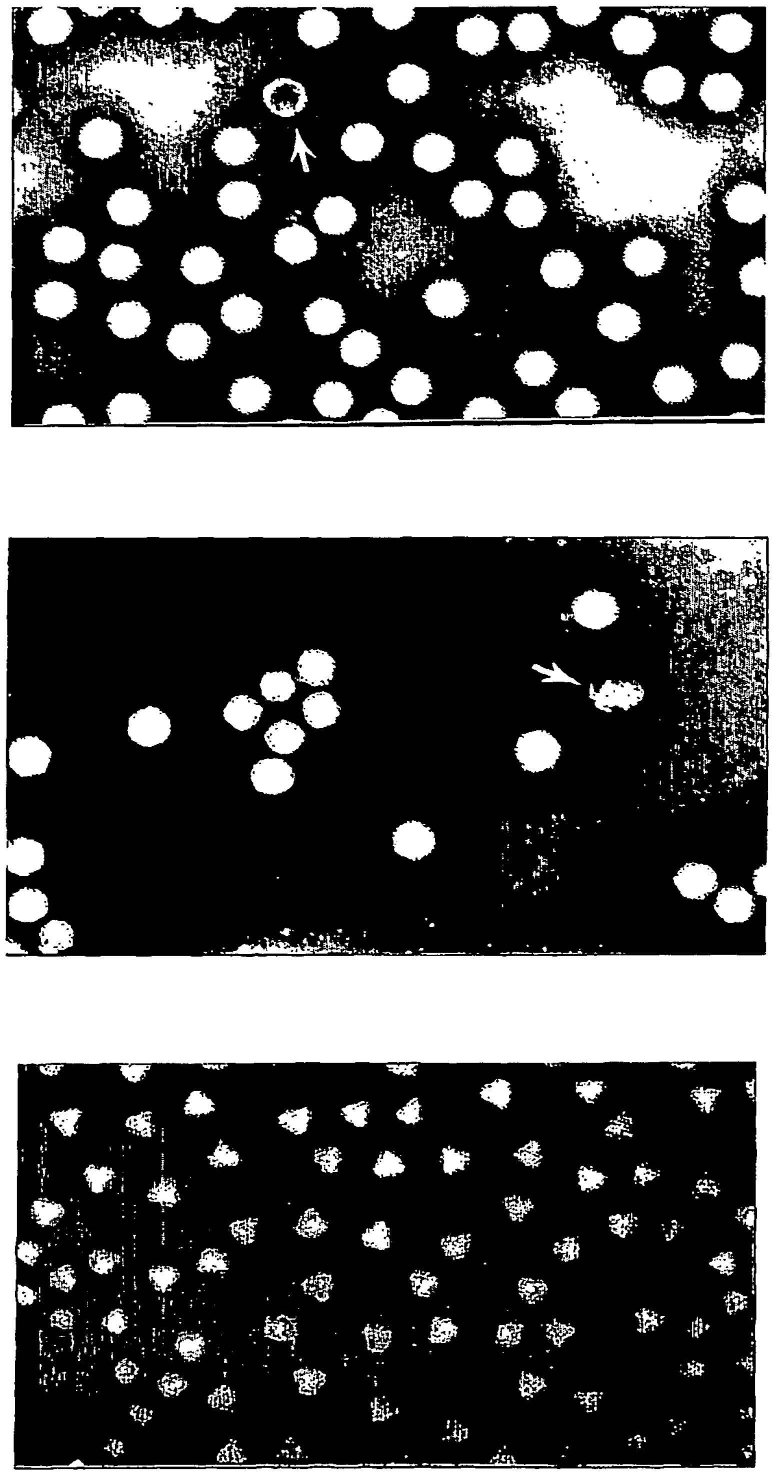

K562
CD34+
NEGATIVE CONTROL
CAR
α_V- INTEGRINS
COUNTS
1%
15%
77%
1%
5.9%
17%
M1

FIG. 10-ii



Ad 35

Ad 9

Ad 5

FIG. 11

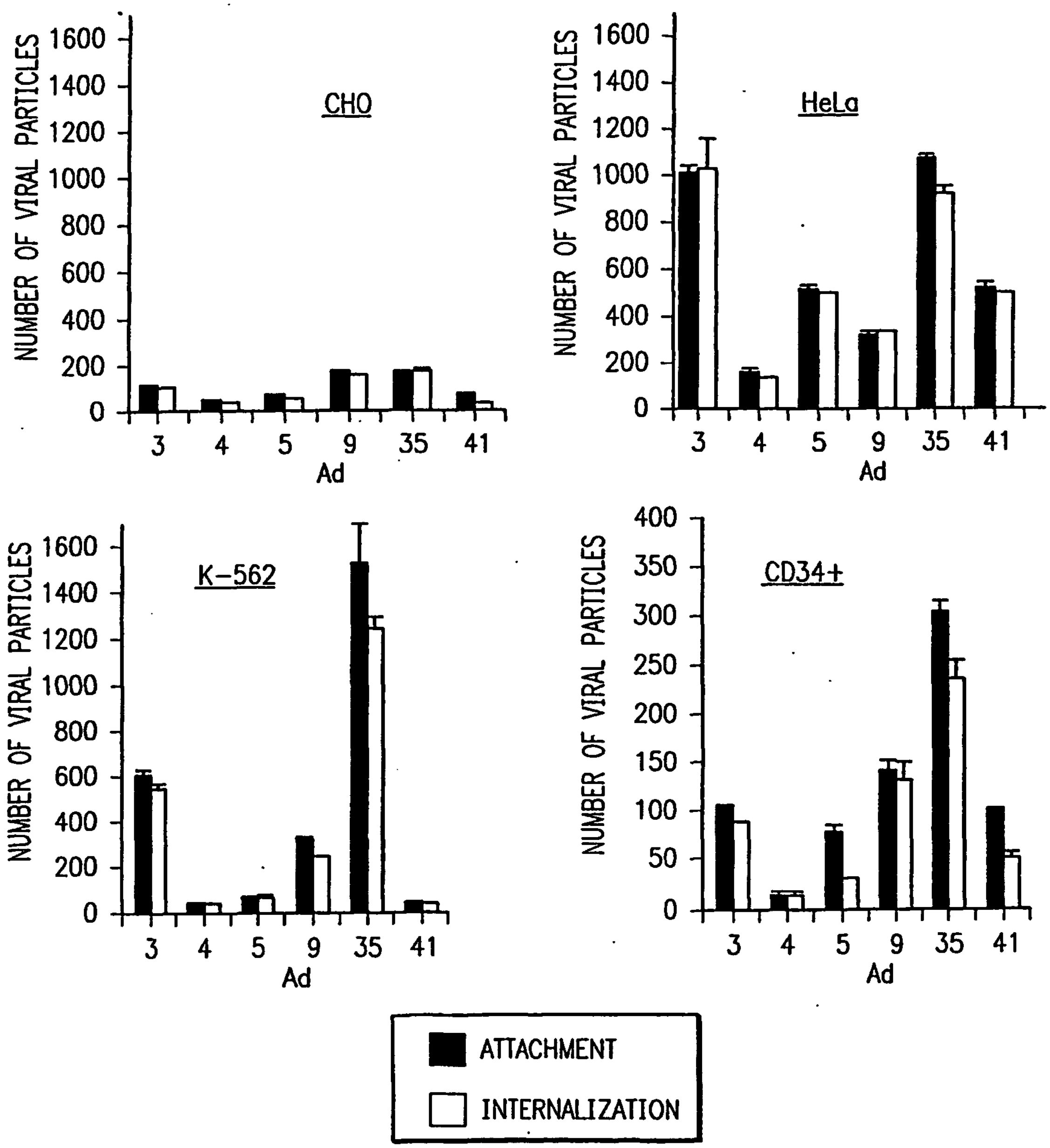

NUMBER OF VIRAL PARTICLES
CHO
HeLa
K-562
CD34+
Ad
ATTACHMENT
INTERNALIZATION

FIG. 12

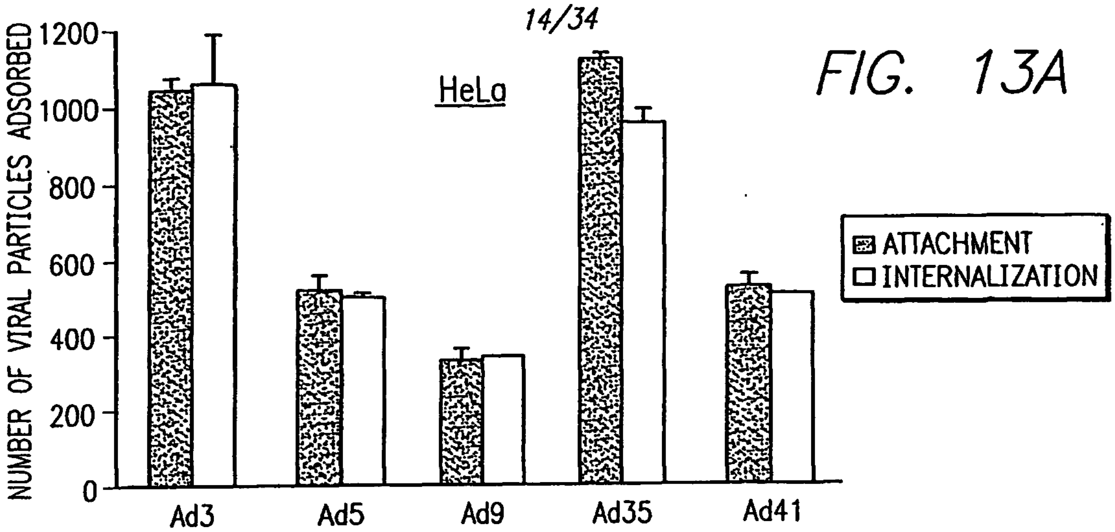


FIG. 13A

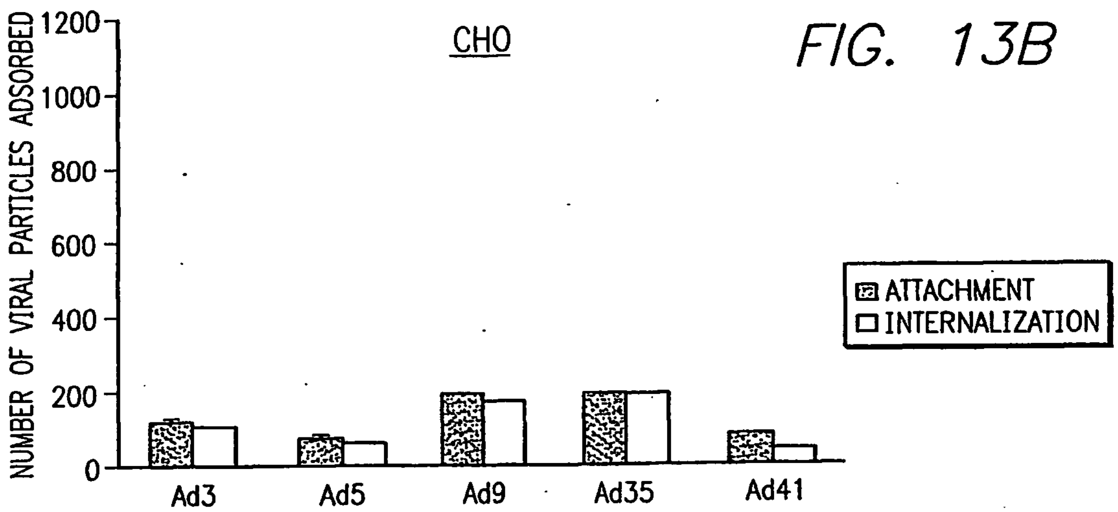


FIG. 13B

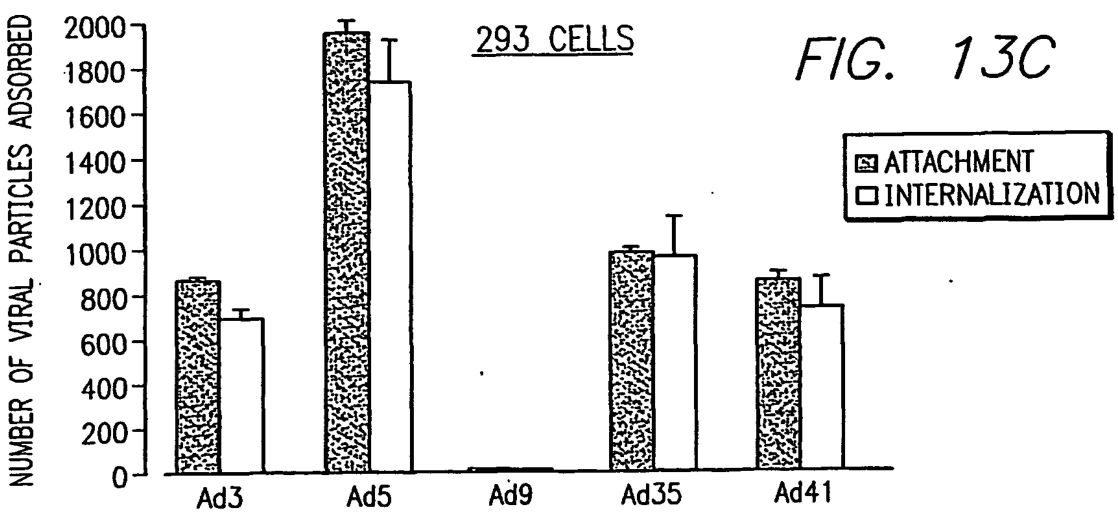


FIG. 13C

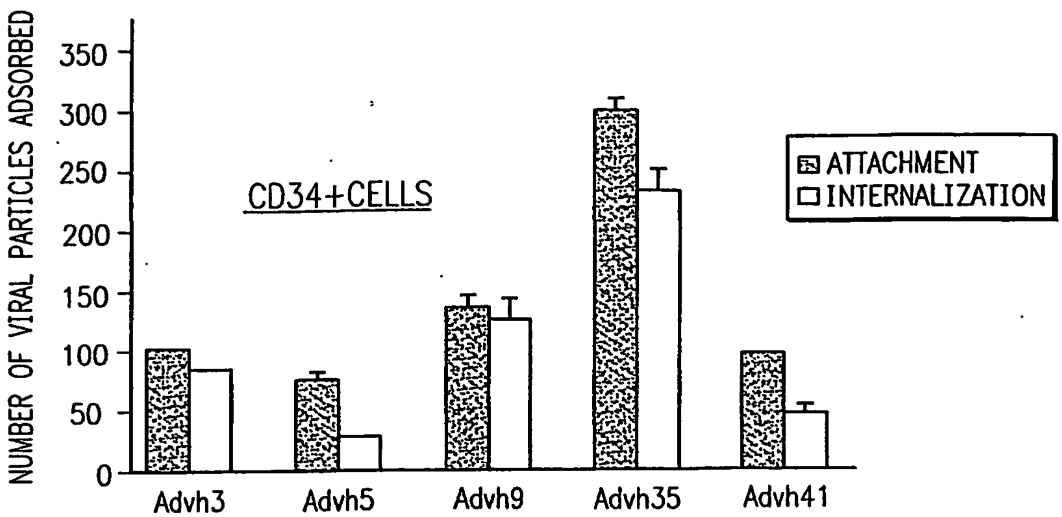


FIG. 14A

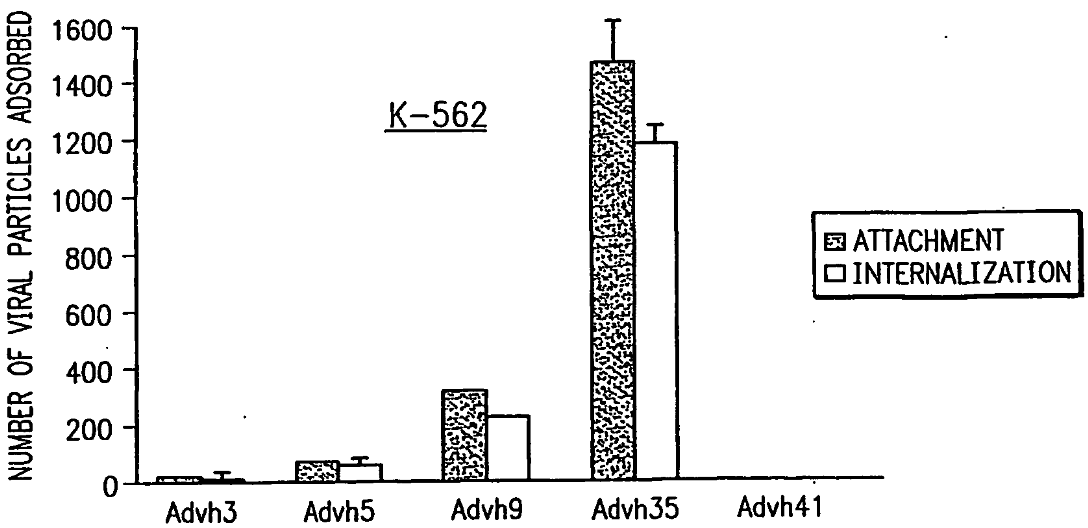


FIG. 14B

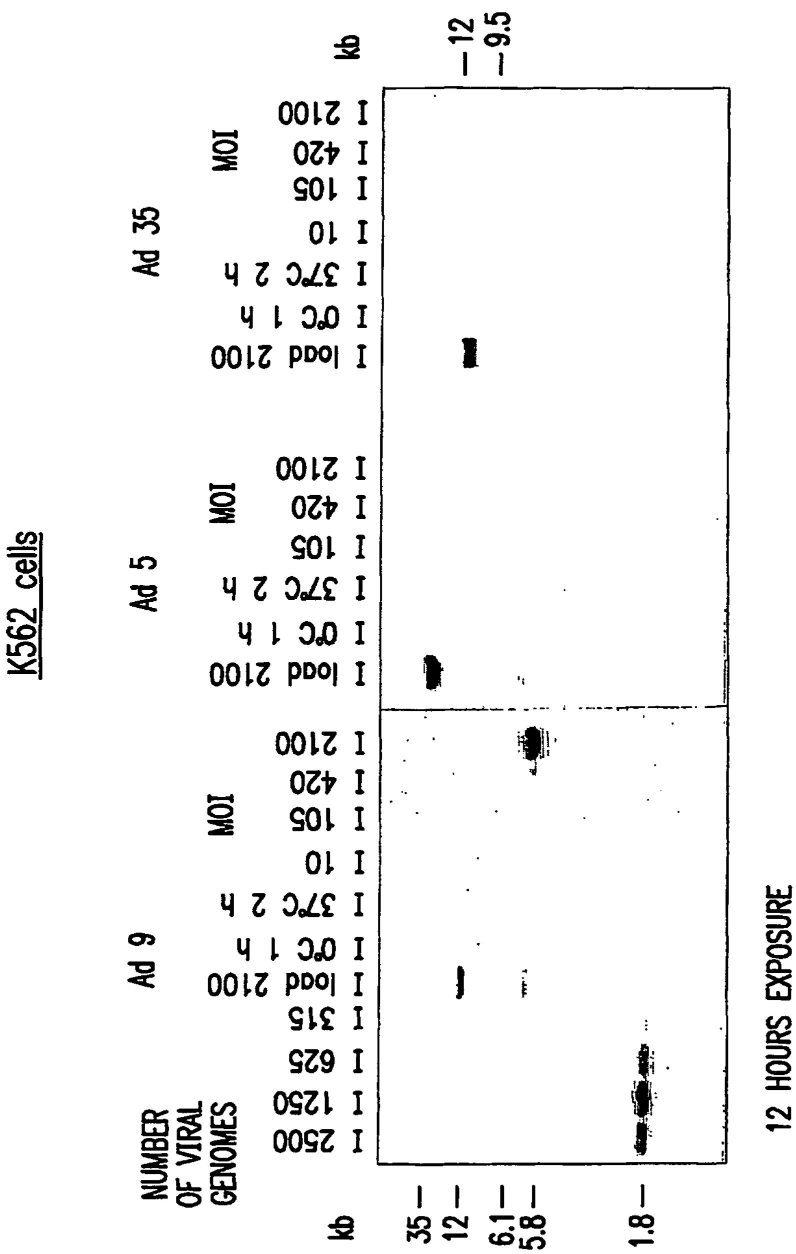

K562 cells
NUMBER OF VIRAL GENOMES
2500
1250
625
315
Ad 9
load 2100
0°C 1 h
37°C 2 h
10
105
420
2100
MOI
Ad 5
load 2100
0°C 1 h
37°C 2 h
105
420
2100
MOI
Ad 35
load 2100
0°C 1 h
37°C 2 h
10
105
420
2100
MOI
kb
35
12
6.1
5.8
1.8
kb
12
9.5
12 HOURS EXPOSURE

FIG. 15A

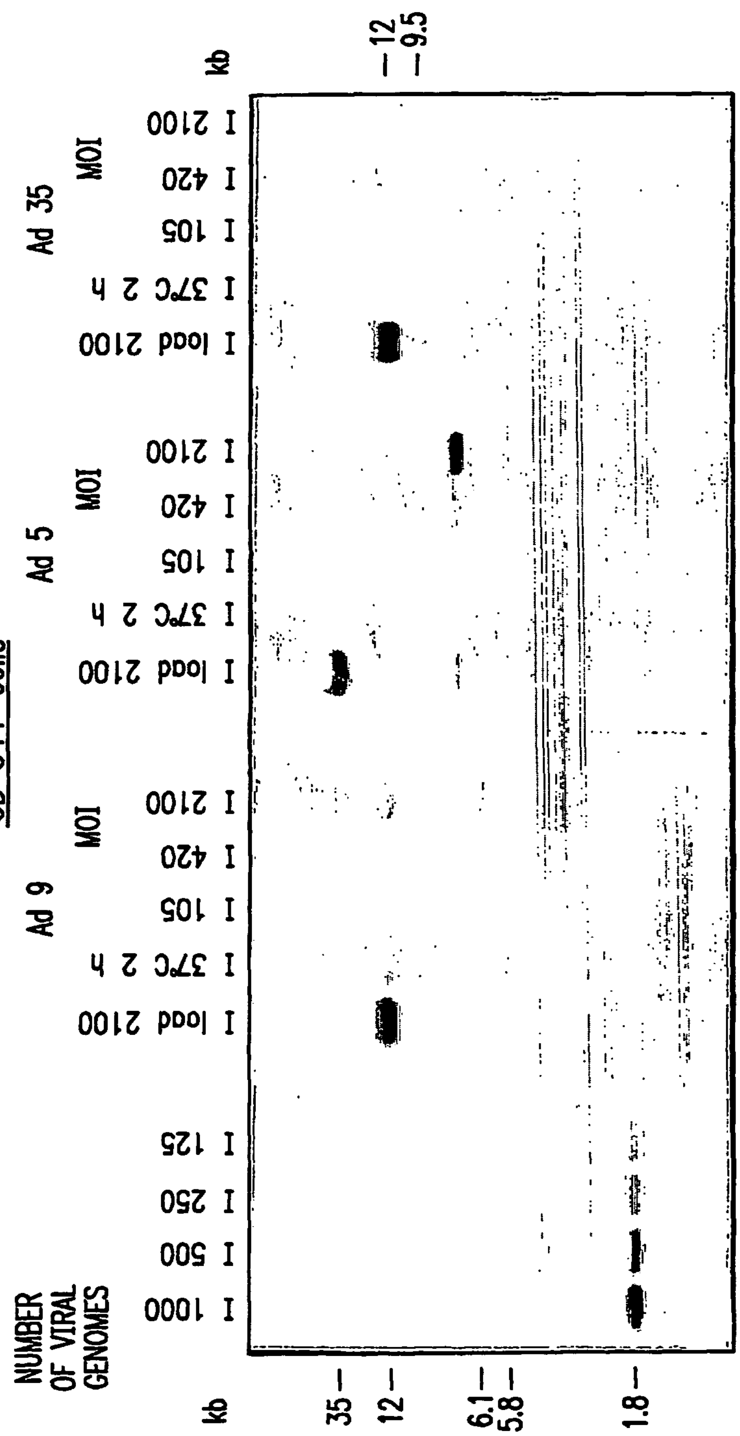


FIG. 15B

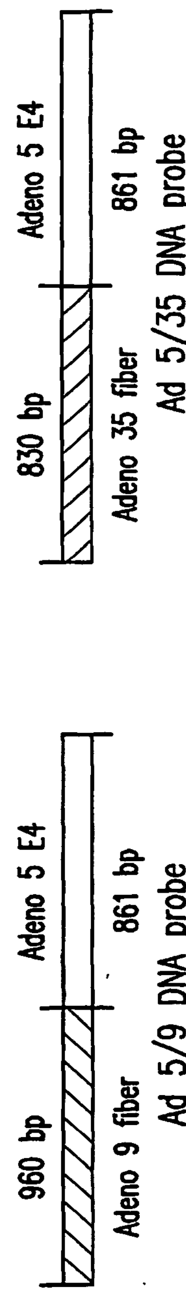


FIG. 15C

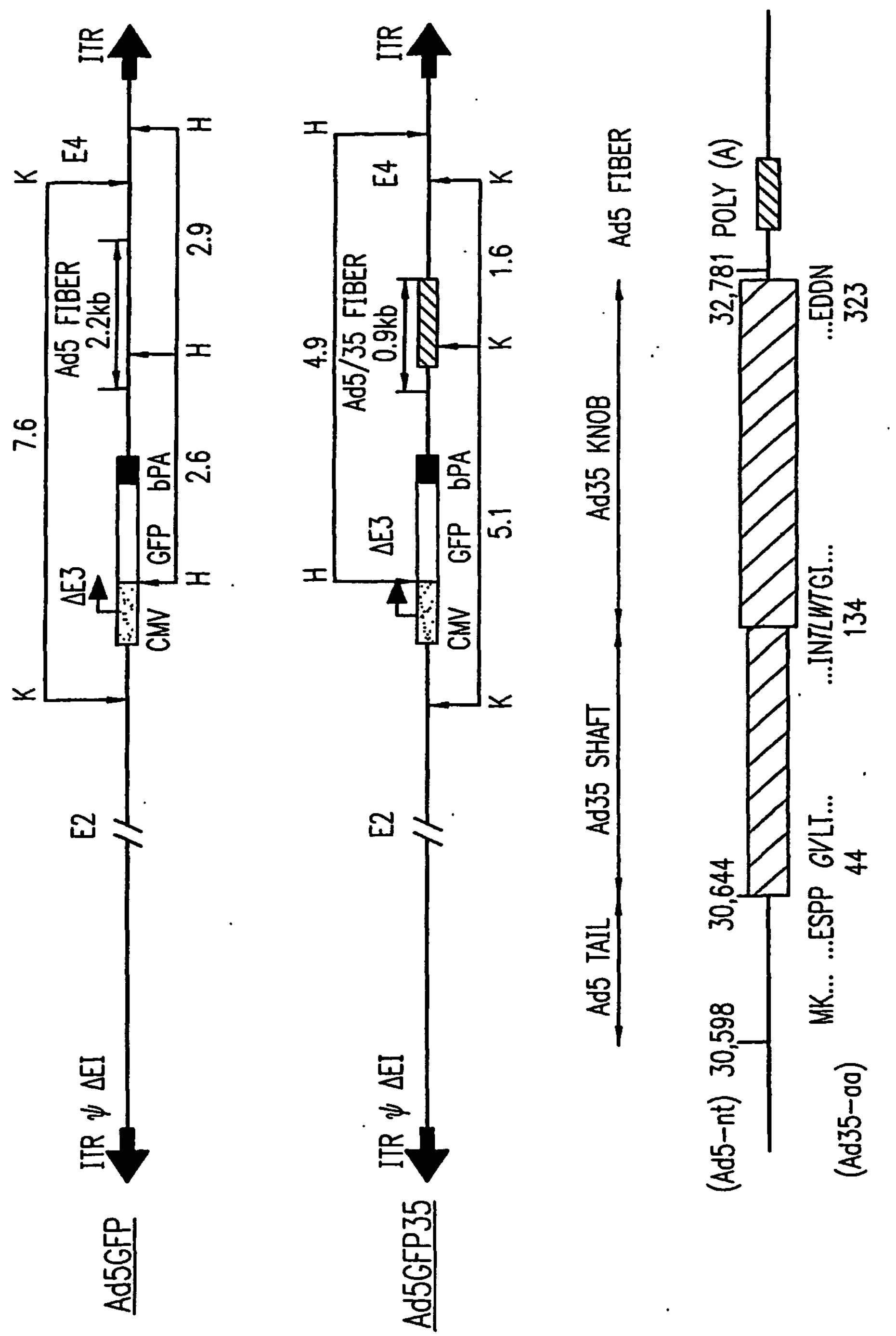

Ad5GFP
ITR ψ ΔEI
E2
K
7.6
K
CMV
ΔE3
GFP
bPA
H
2.6
Ad5 FIBER
2.2kb
H
2.9
E4
H
ITR
Ad5GFP35
ITR ψ ΔEI
E2
K
CMV
GFP
bPA
ΔE3
H
5.1
4.9
Ad5/35 FIBER
0.9kb
K
1.6
E4
K
H
ITR
Ad5 TAIL
Ad35 SHAFT
Ad35 KNOB
Ad5 FIBER
(Ad5-nt) 30,598
30,644
32,781
POLY (A)
(Ad35-aa)
MK...
...ESPP GVLT...
44
...INTLWTGI...
134
...EDDN
323

FIG. 16A

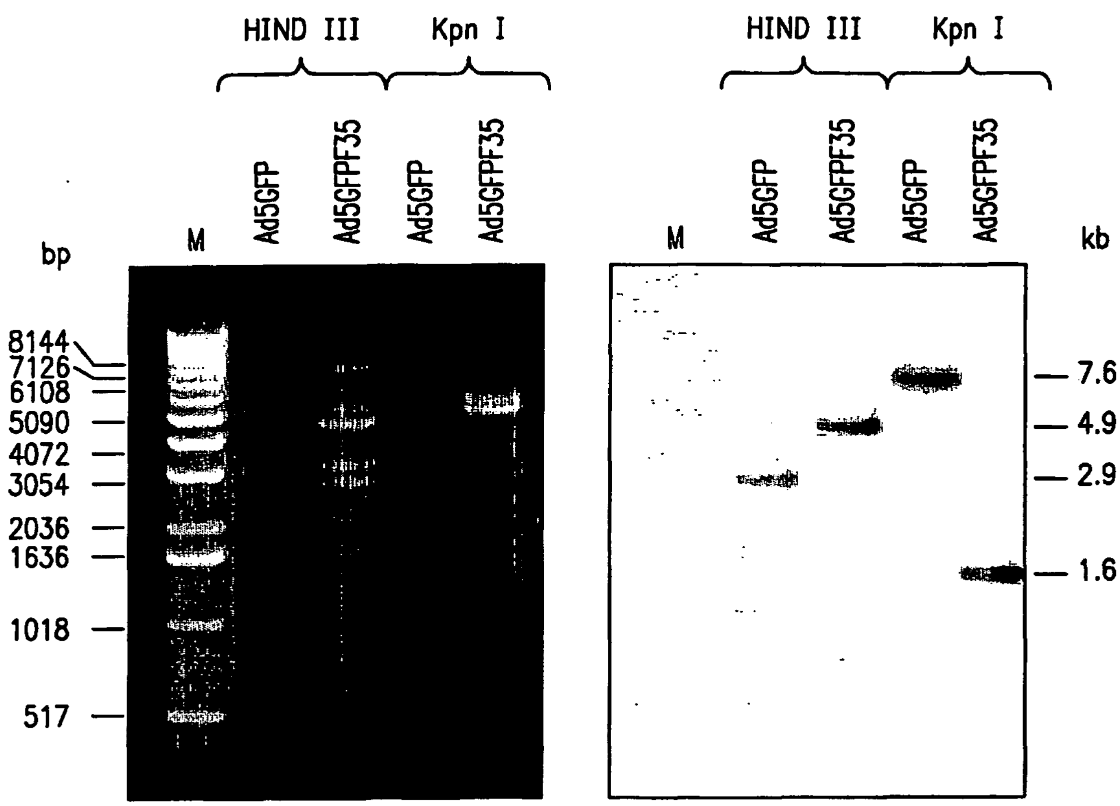

HIND III
Kpn I
M
Ad5GFP
Ad5GFPF35
Ad5GFP
Ad5GFPF35
bp
8144
7126
6108
5090
4072
3054
2036
1636
1018
517
HIND III
Kpn I
M
Ad5GFP
Ad5GFPF35
Ad5GFP
Ad5GFPF35
kb
7.6
4.9
2.9
1.6

FIG. 16B

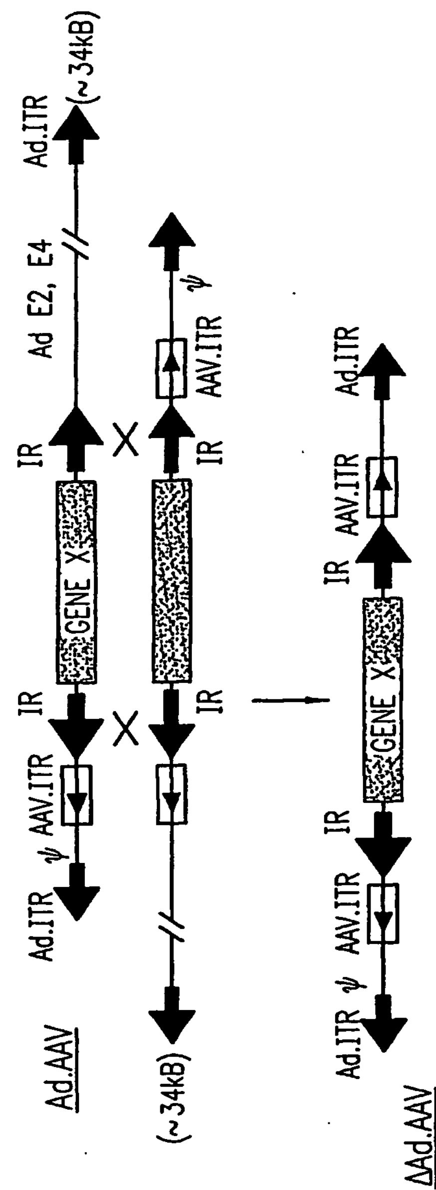


FIG. 17A

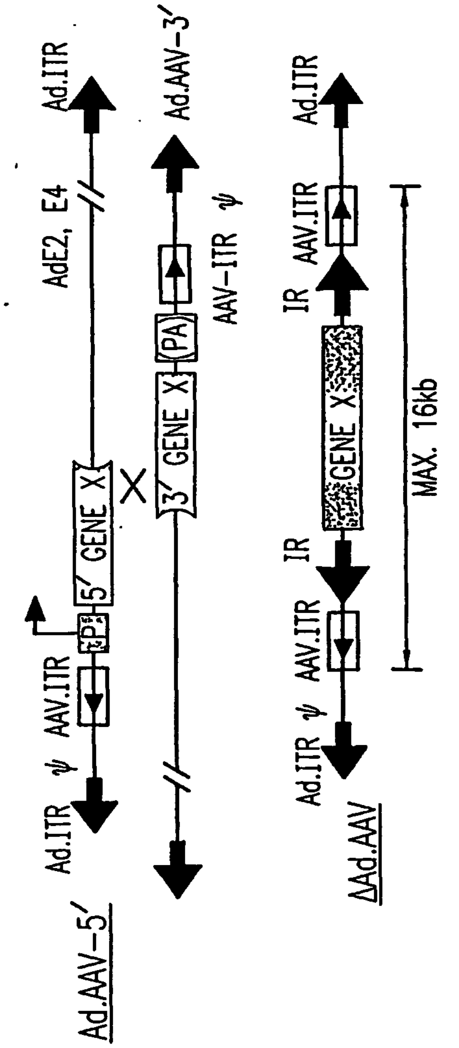


FIG. 17B

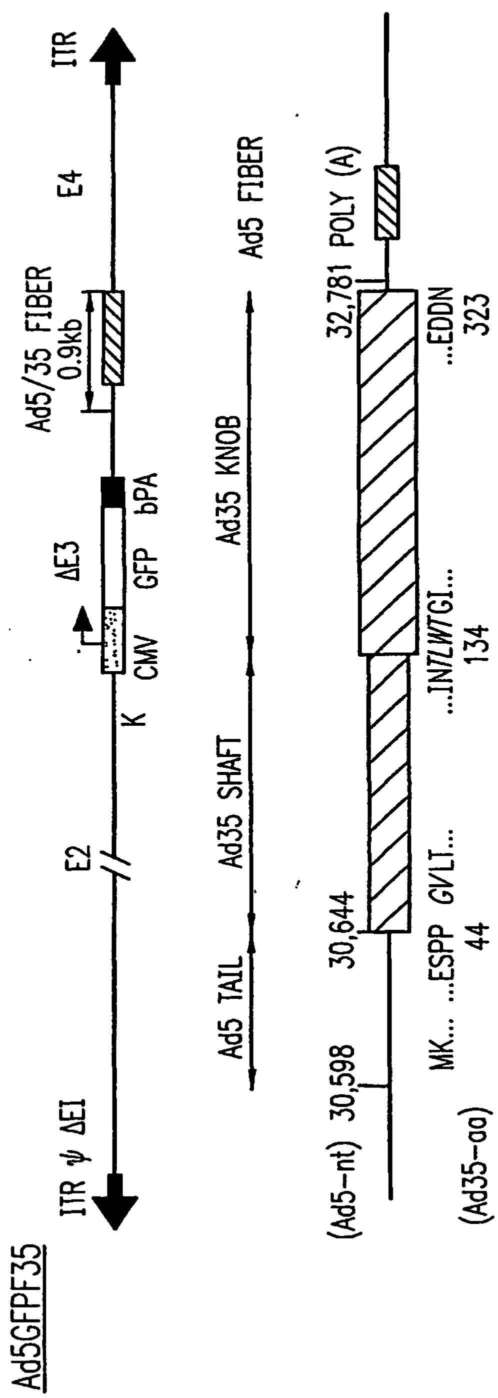

Ad5GFPF35
ITR ψ ΔEI
E2
K
CMV
GFP
bPA
ΔE3
Ad5/35 FIBER
0.9kb
E4
ITR
Ad5 TAIL
Ad35 SHAFT
Ad35 KNOB
Ad5 FIBER
(Ad5-nt)
30,598
30,644
32,781
POLY (A)
(Ad35-aa)
MK...
...ESPP
44
GVLT...
...INTLWTGI...
134
...EDDN
323

FIG. 18

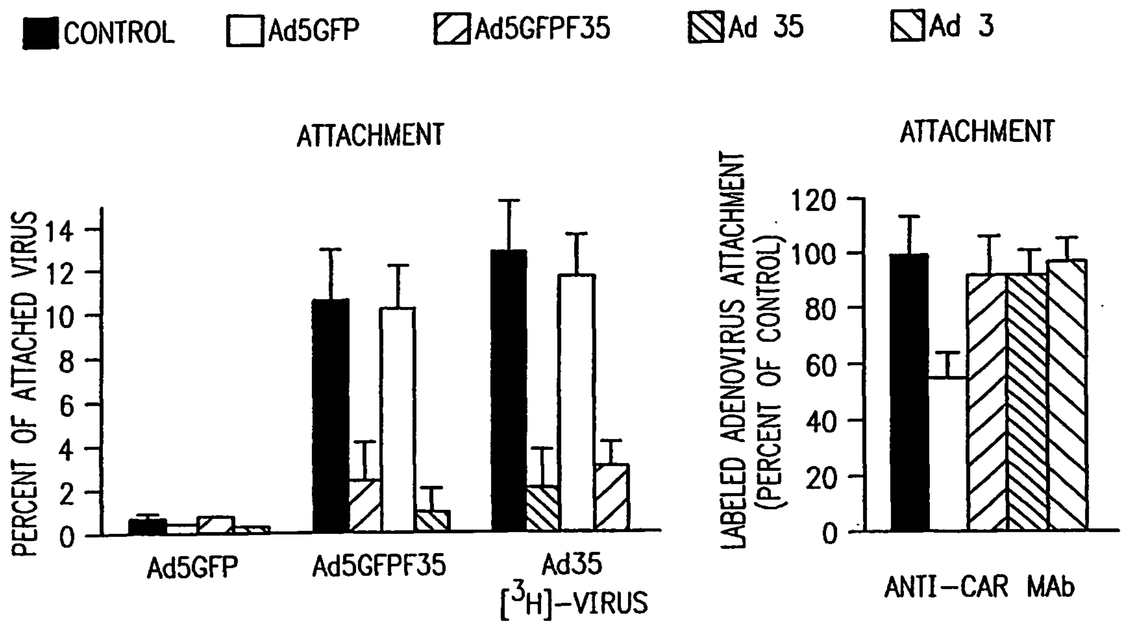


FIG. 19A FIG. 19B

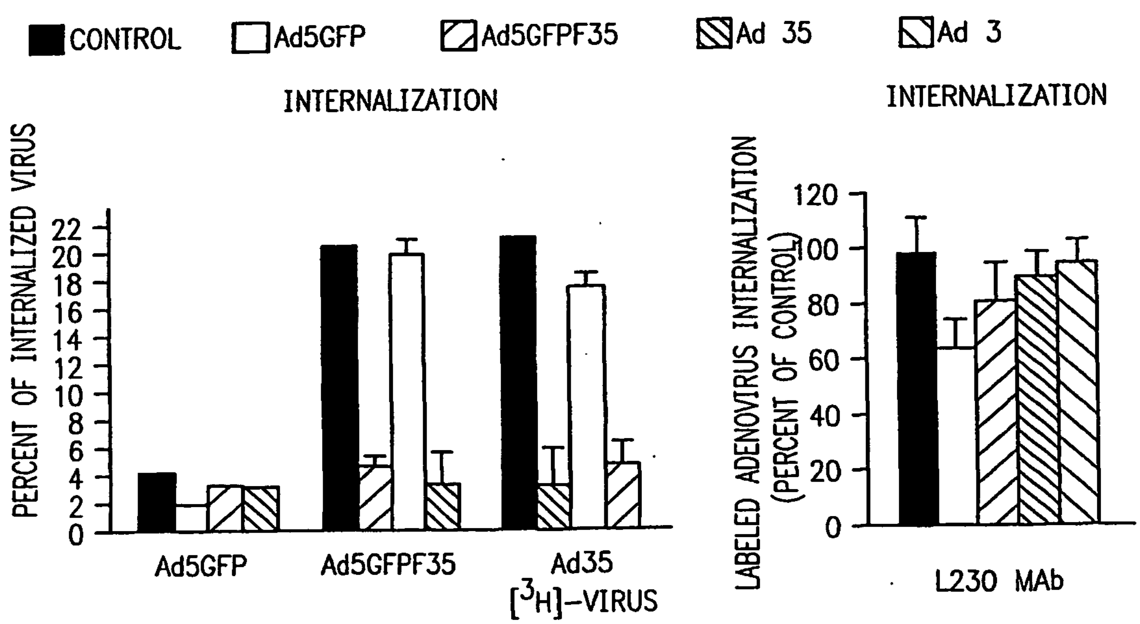


FIG. 19C FIG. 19D

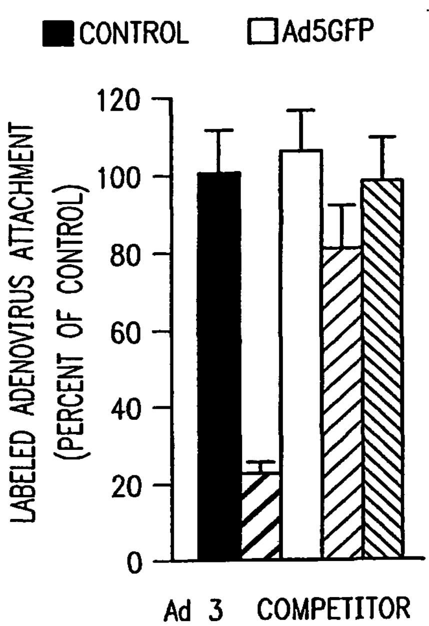


FIG. 20A

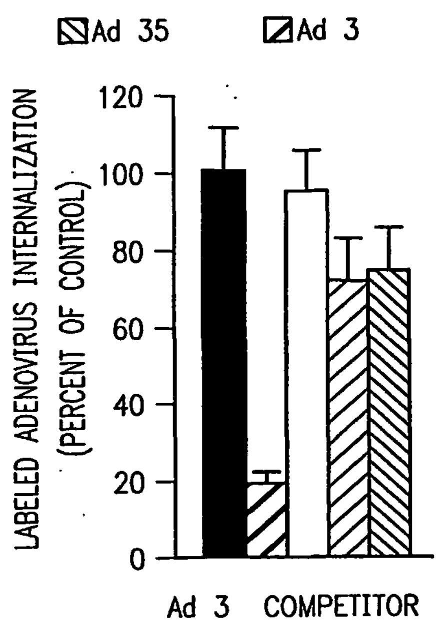


FIG. 20B

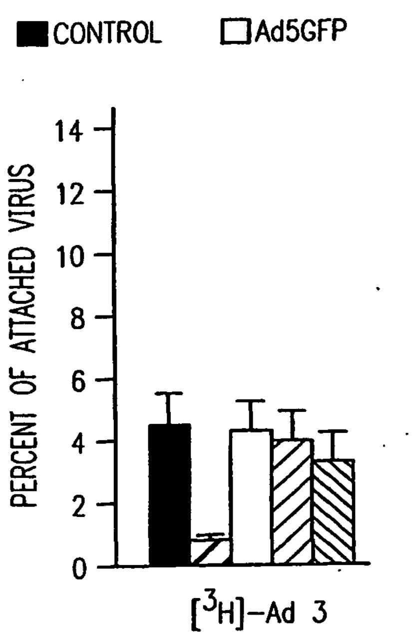


FIG. 20C

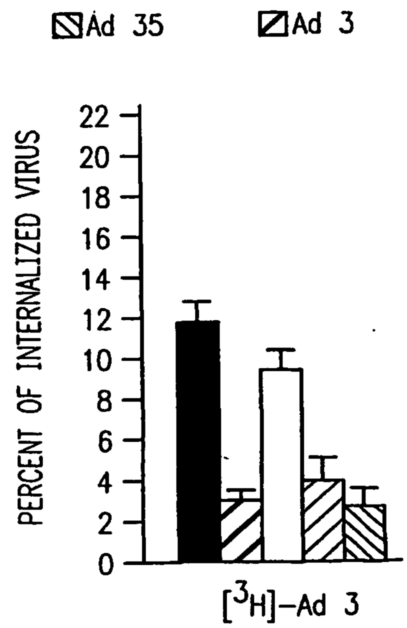
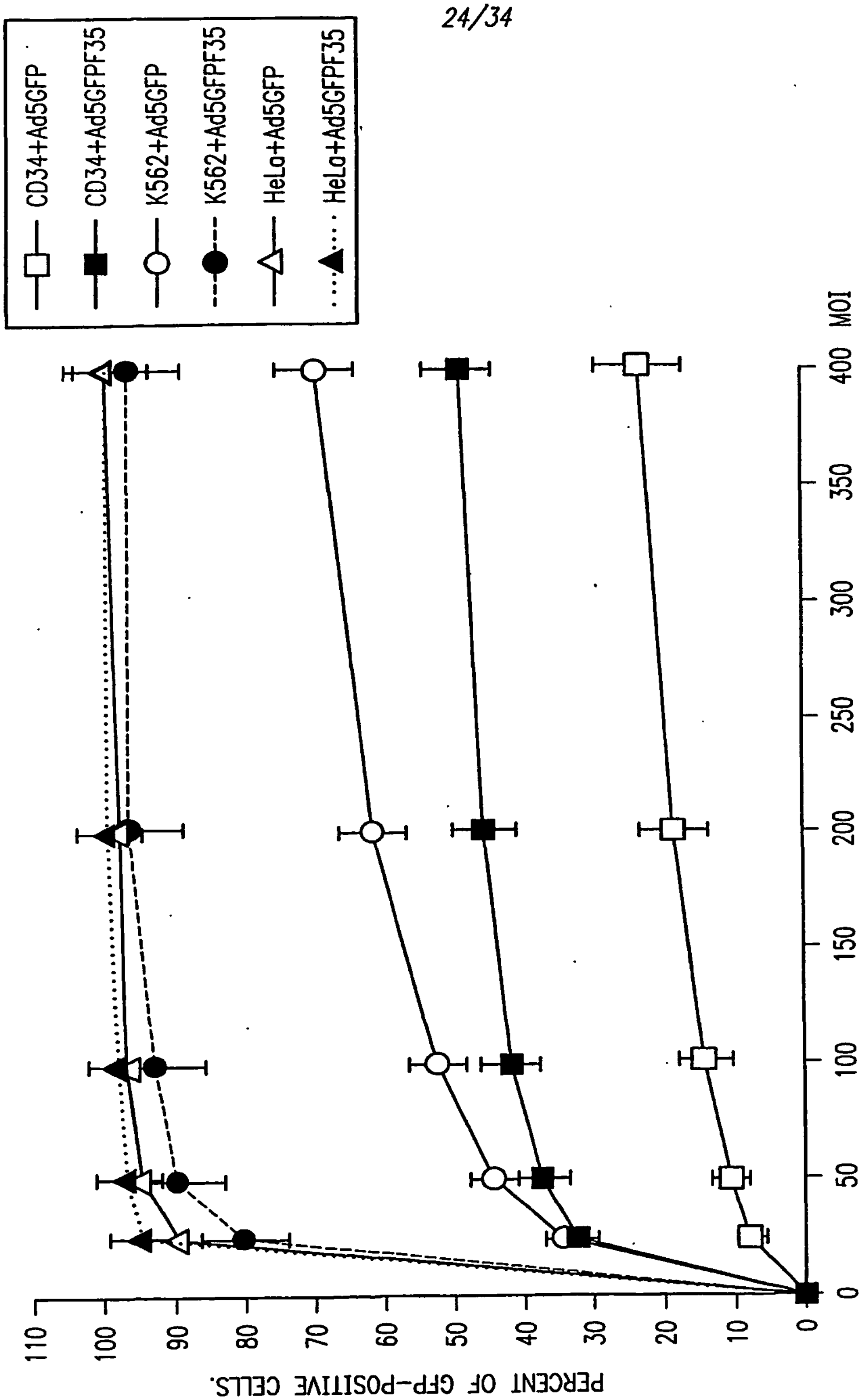


FIG. 20D



CD34+Ad5GFP
CD34+Ad5GFPF35
K562+Ad5GFP
K562+Ad5GFPF35
HeLa+Ad5GFP
HeLa+Ad5GFPF35
PERCENT OF GFP-POSITIVE CELLS.
0
10
20
30
40
50
60
70
80
90
100
110
0
50
100
150
200
250
300
350
400
MOI

FIG. 21

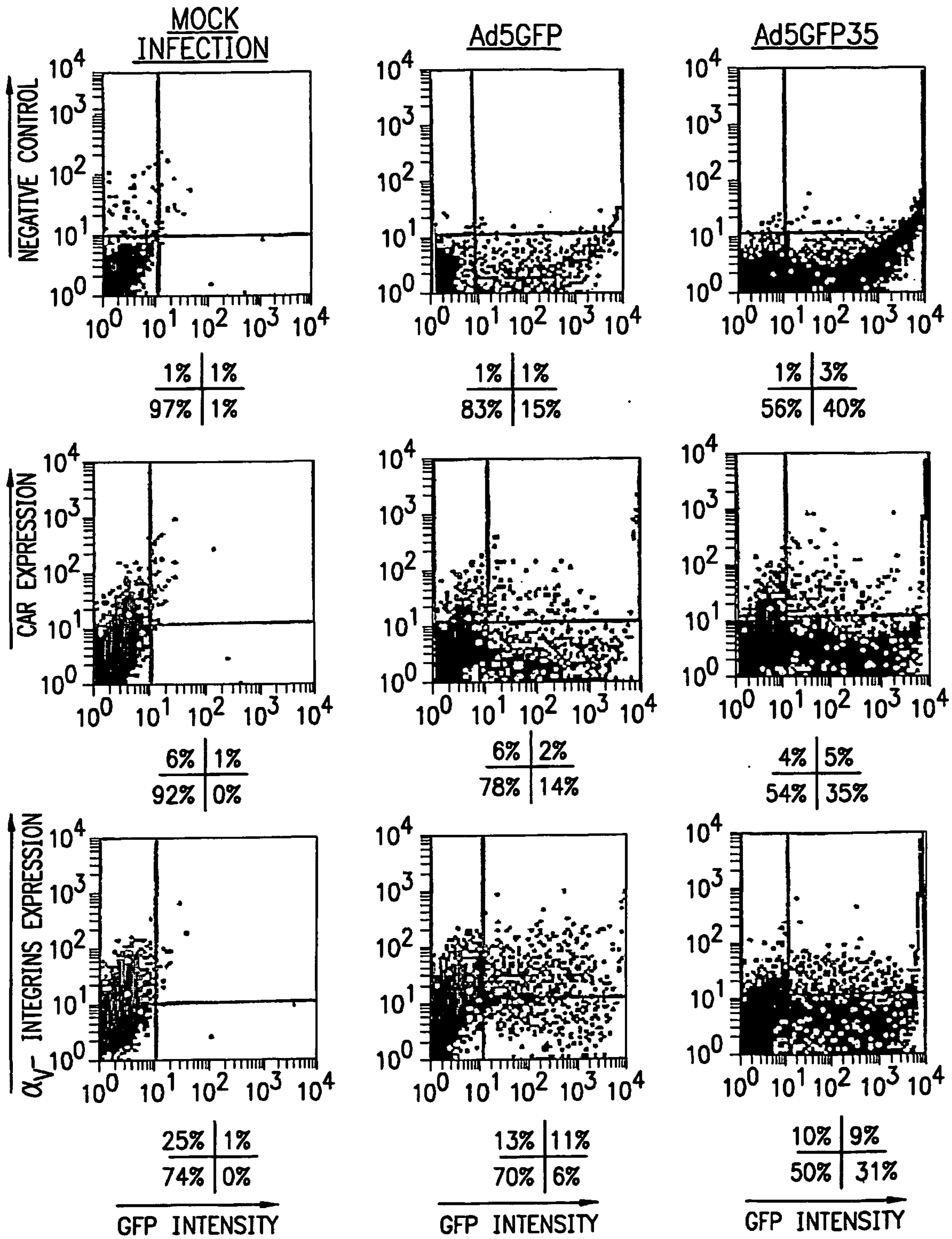

MOCK INFECTION
Ad5GFP
Ad5GFP35
NEGATIVE CONTROL
CAR EXPRESSION
α_V- INTEGRINS EXPRESSION
10^0 10^1 10^2 10^3 10^4
1% 1% 97% 1%
1% 1% 83% 15%
1% 3% 56% 40%
6% 1% 92% 0%
6% 2% 78% 14%
4% 5% 54% 35%
25% 1% 74% 0%
13% 11% 70% 6%
10% 9% 50% 31%
GFP INTENSITY
GFP INTENSITY
GFP INTENSITY

FIG. 22

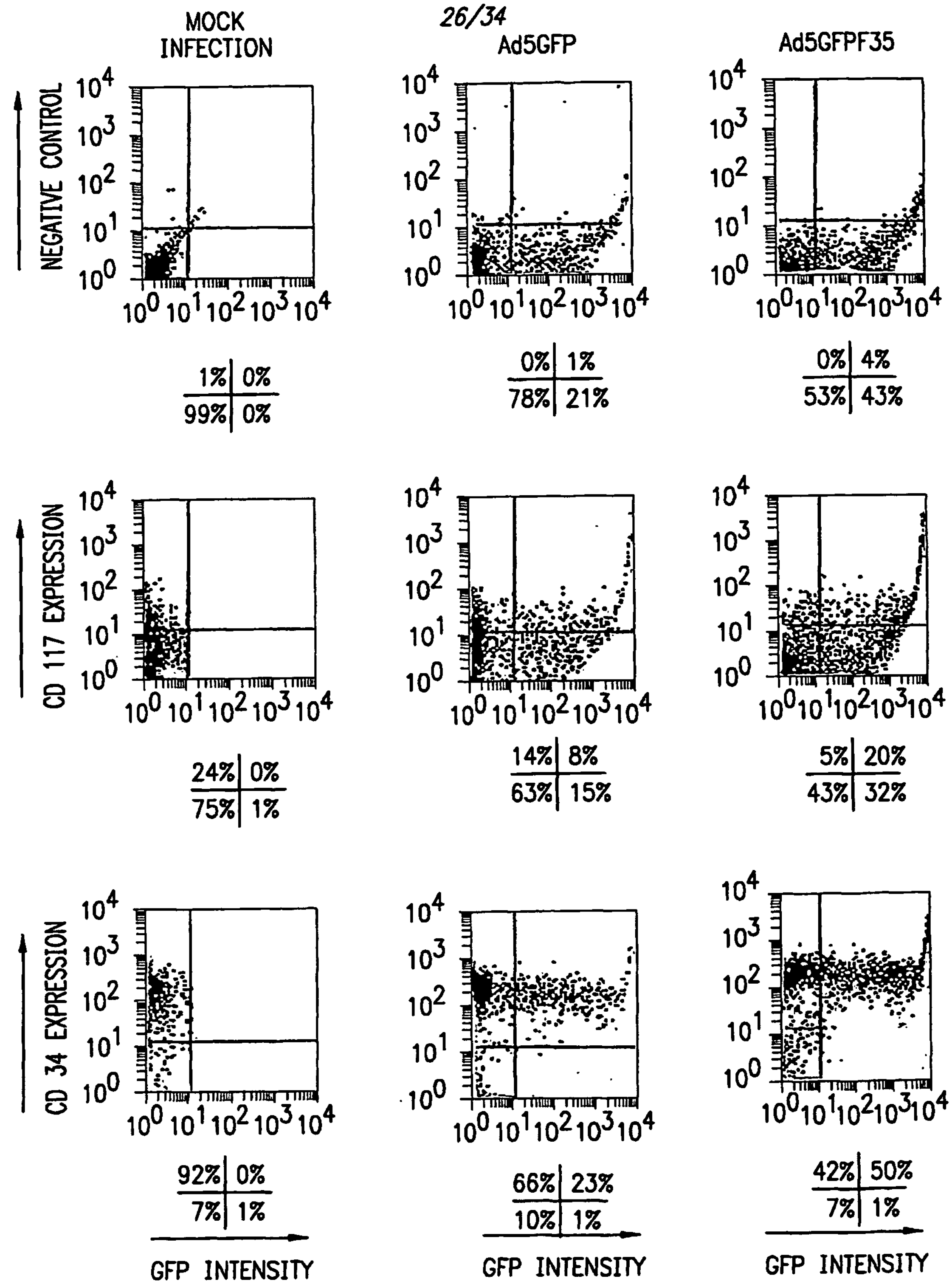

MOCK INFECTION
Ad5GFP
Ad5GFPF35
NEGATIVE CONTROL
CD 117 EXPRESSION
CD 34 EXPRESSION
10^0 10^1 10^2 10^3 10^4
1% 0% 99% 0%
0% 1% 78% 21%
0% 4% 53% 43%
24% 0% 75% 1%
14% 8% 63% 15%
5% 20% 43% 32%
92% 0% 7% 1%
66% 23% 10% 1%
42% 50% 7% 1%
GFP INTENSITY
GFP INTENSITY
GFP INTENSITY

FIG. 23A

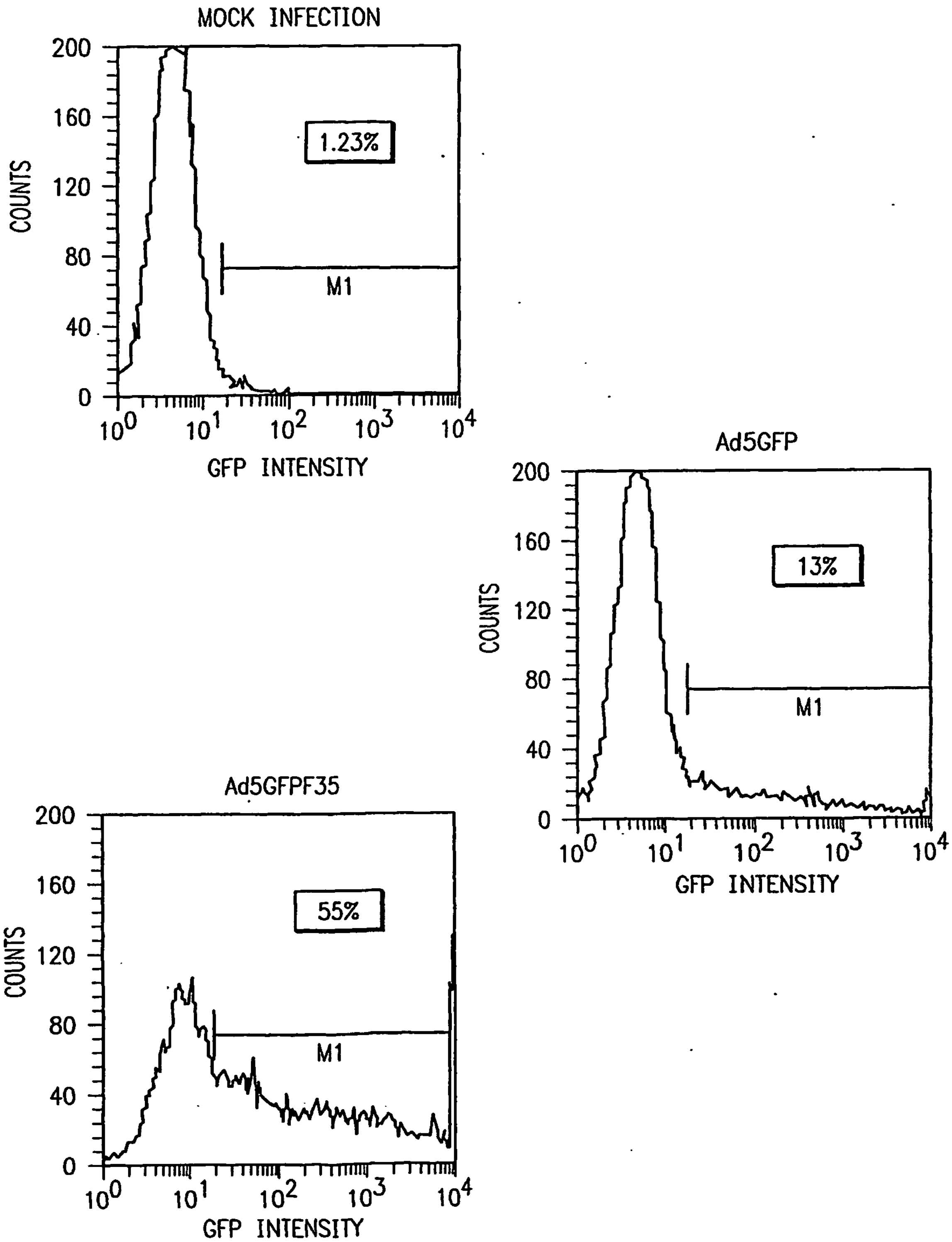

MOCK INFECTION
200
160
120
80
40
0
COUNTS
1.23%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY
Ad5GFP
200
160
120
80
40
0
COUNTS
13%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY
Ad5GFPF35
200
160
120
80
40
0
COUNTS
55%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY

FIG. 23B

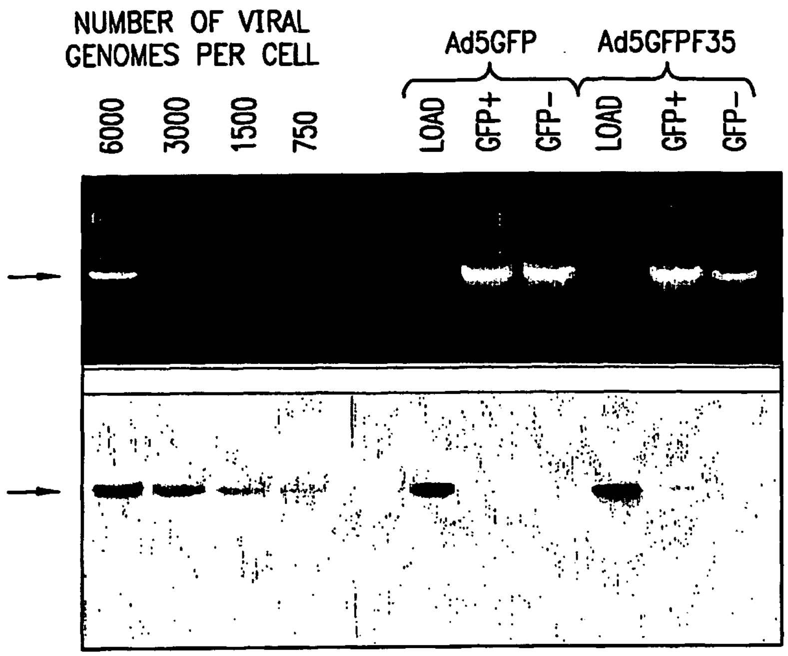


FIG. 24A

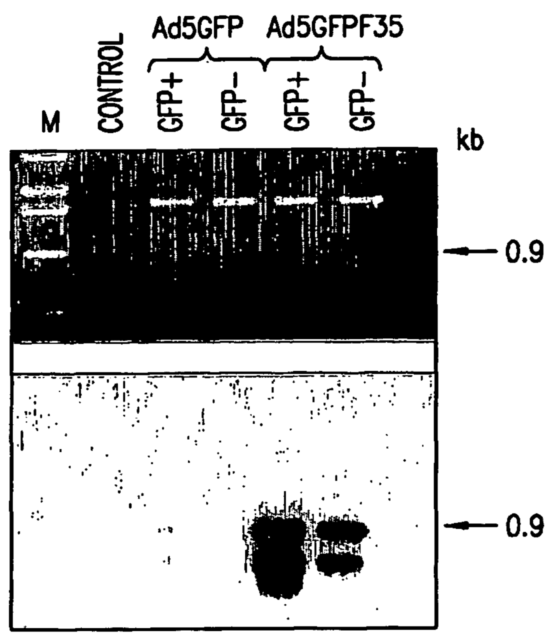
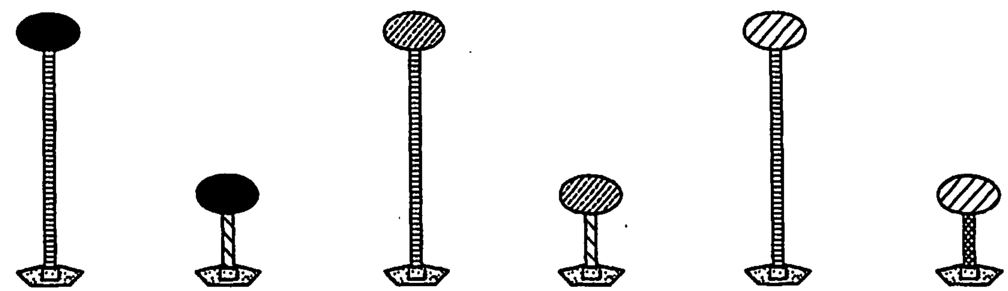


FIG. 24B

STRUCTURE AND FEATURES OF CHIMERIC ADENOVIRUS VECTORS.



Adeno vector	Ad 5L	Ad 5S	Ad 5/9L	Ad 5/9S	Ad 5/35L	Ad 5/35S
Binding to CAR	YES	YES	YES	YES	NO	NO
genome/PFU ratio	13	143	29	582	17	14
Attachment to/internalization into						
293	+++	++	++	+/−−	+++	+++
K562	−/+	−−/+	−−/+	−−−	+++	+++
Y79	++	+	+	+/−	+	+
Gene transfer efficiency						
293	+++	++	++	+/−−	+++	+++
K562	−/+	−−/+	−−/+	−−−	+++	+++
Y79	++	+	+	+/−	+	+

FIG. 25

Top View of Adenovirus Type 5 Fiber Knob Domain

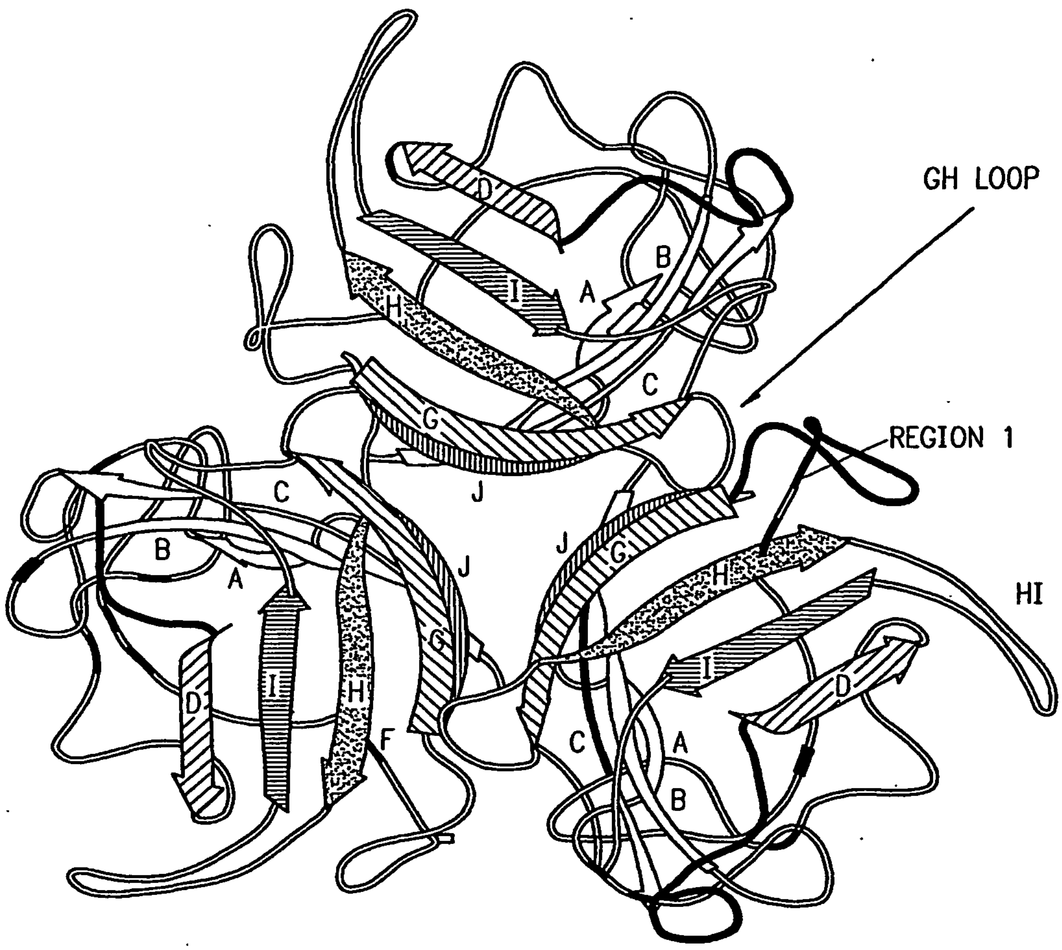


FIG. 26

Amino Acid Composition of Wt and Modified Adeno 5 GH-loop

Adeno 5 wt GH-loop	V Y]L N G D K T K [P V
Adeno 5/9 GH-loop chimera	V Y]L G G K P D Q [P V
Adeno 5 GH-Cys 1	V Y]L N G C G S C [P V
Adeno 5 GH-Cys 2	V Y]L N G C G S G C [P V
Adeno 5 GH-peptide	V Y]L N G C G $(X)^{10}$ G C [P V

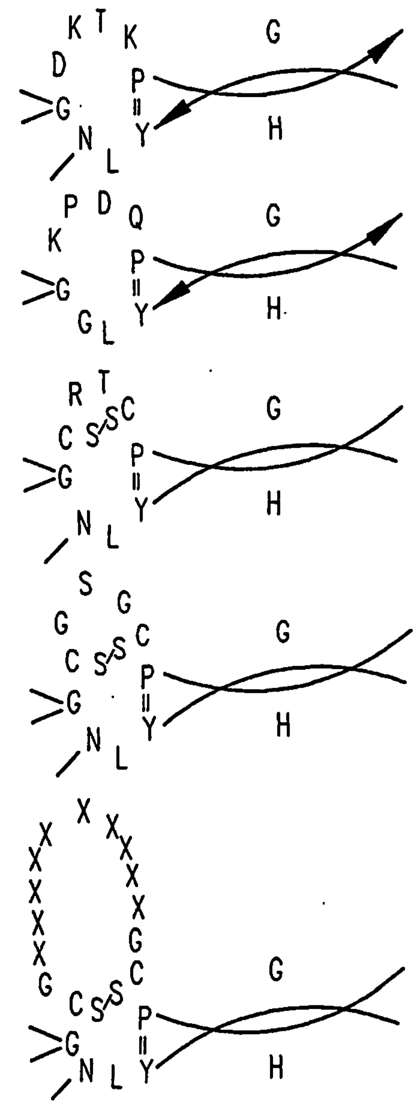


FIG. 27

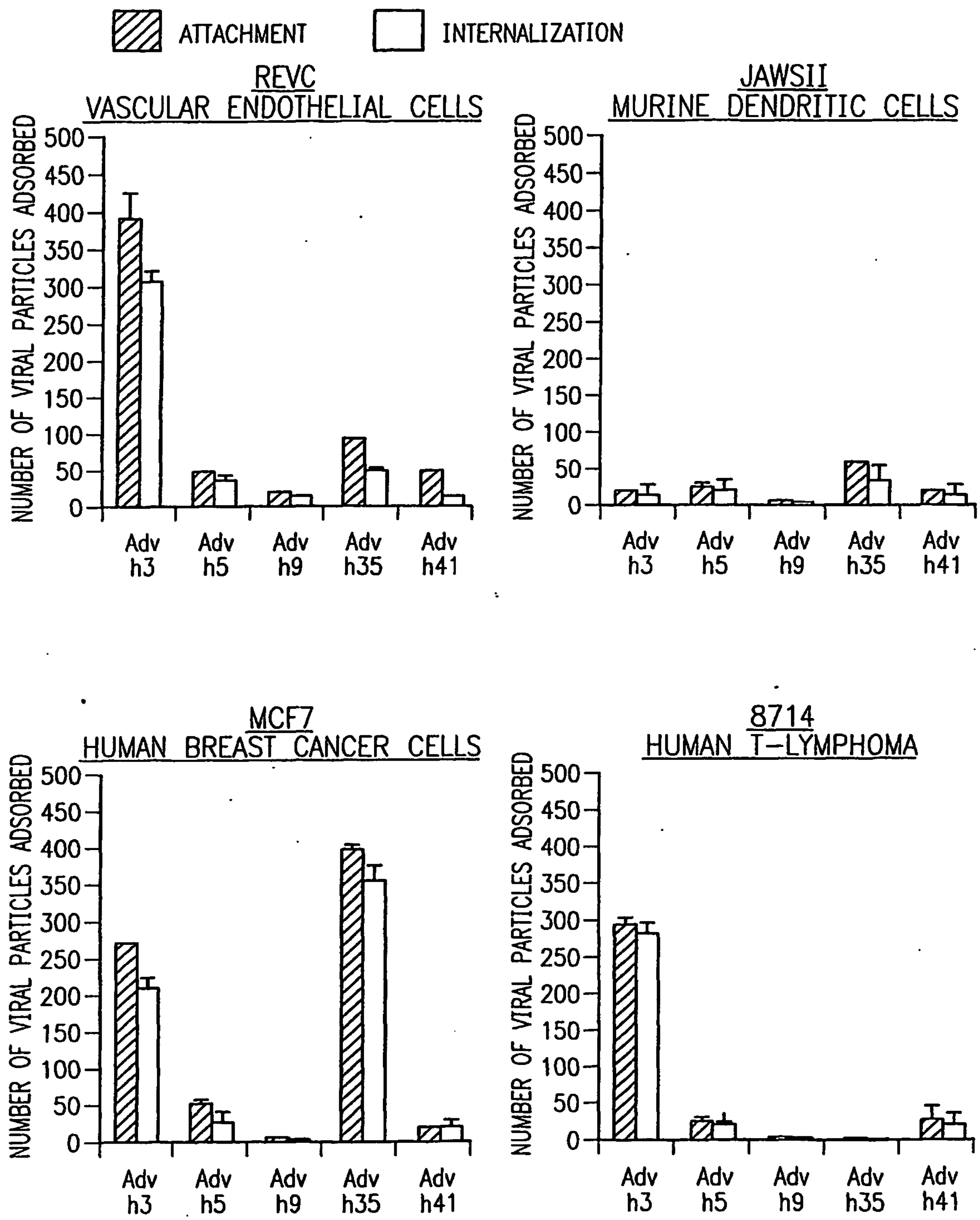

ATTACHMENT
INTERNALIZATION
REVC
VASCULAR ENDOTHELIAL CELLS
JAWSII
MURINE DENDRITIC CELLS
MCF7
HUMAN BREAST CANCER CELLS
8714
HUMAN T-LYMPHOMA
NUMBER OF VIRAL PARTICLES ADSORBED
0
50
100
150
200
250
300
350
400
450
500
Adv h3
Adv h5
Adv h9
Adv h35
Adv h41

FIG. 28

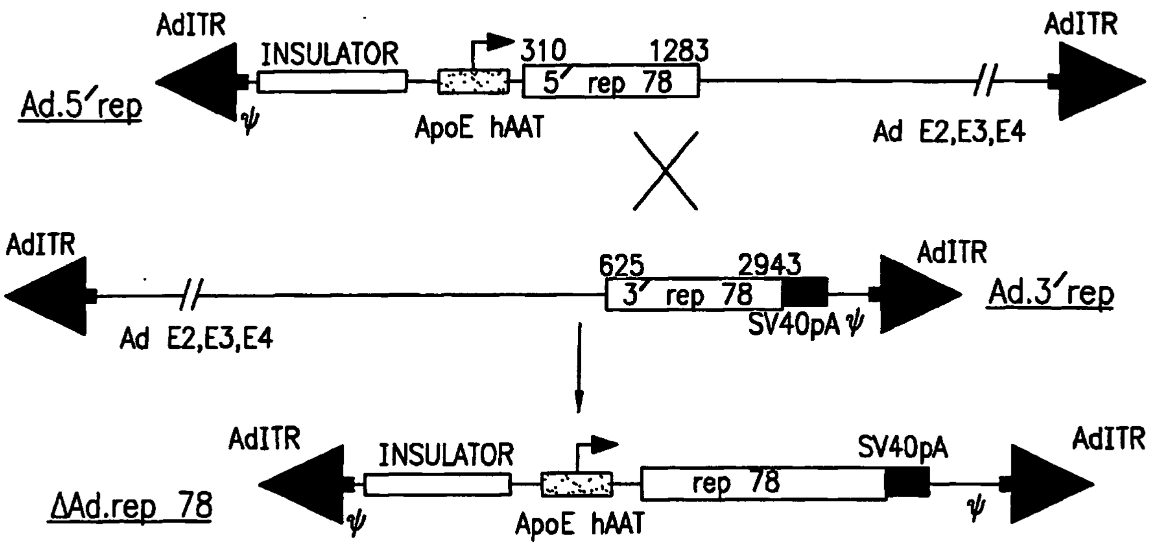


FIG. 29A

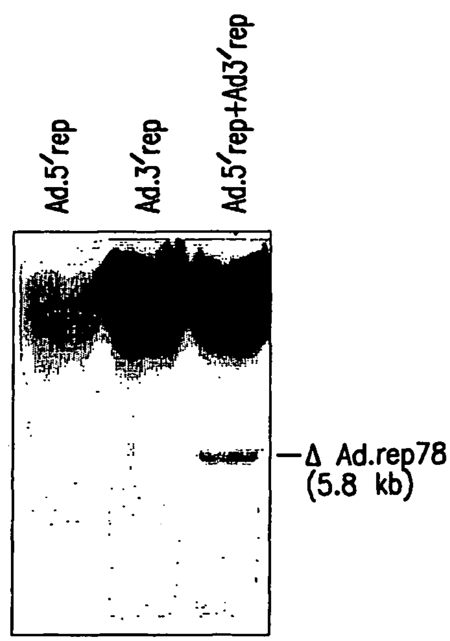


FIG. 29B

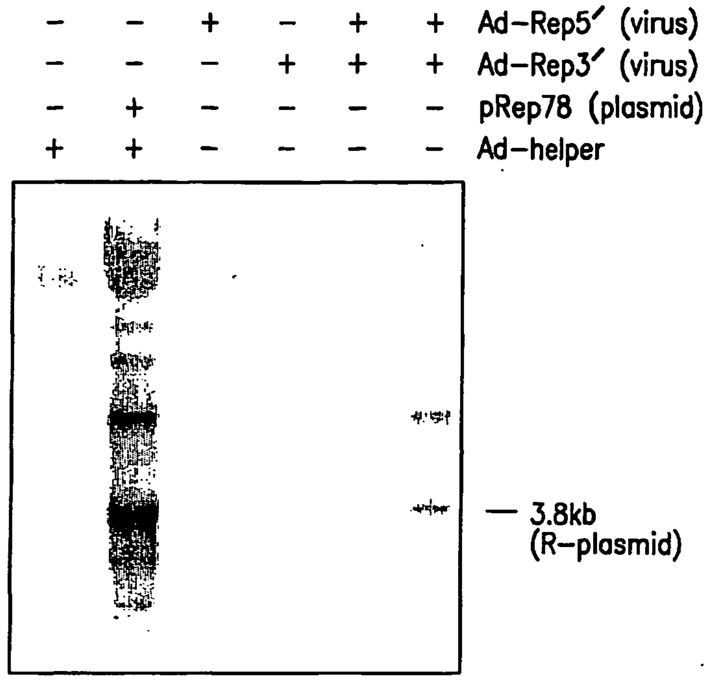


FIG. 29C

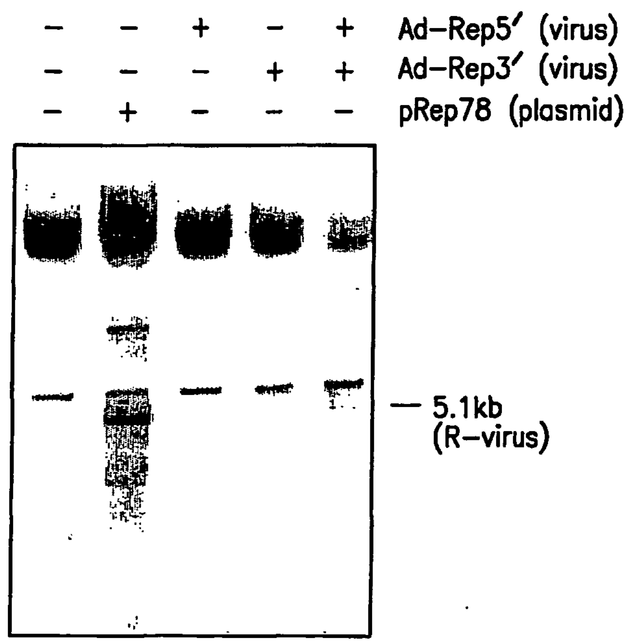


FIG. 29D

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 00/15442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/861 C12N15/864 C12N15/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 856 152 A (WILSON JAMES M ET AL) 5 January 1999 (1999-01-05)	1,2, 5-11,15, 36-44, 48-54,58
Y	the whole document	3,4,13, 16-32, 35,36, 46,47
Y	WO 98 54346 A (ROELVINK PETRUS W ;EINFELD DAVID (US); GENVEC INC (US); KOVESDI IM) 3 December 1998 (1998-12-03) page 7, line 27 -page 14, line 26 the whole document	3,4, 16-32, 35,56

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2000	30/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern   nal Application No
PCT/US 00/15442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BURCIN M. M. ET AL.: "Adenovirus-mediated regulable target gene expression in vivo" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 96, January 1999 (1999-01), pages 355-360, XP002130326 ISSN: 0027-8424	1,2
Y	the whole document	13,46
X	RECCHIA A. ET AL.: "Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 6, 16 March 1999 (1999-03-16), pages 2615-2620, XP002152917 ISSN: 0027-8424 the whole document	1,2, 5-11,14, 36-44, 47-49,58
X	WO 97 38723 A (BARBER JACK ;IMMUSOL INC (US); LI QI XIANG (US); YU GANG (US); YU) 23 October 1997 (1997-10-23)	55,57
Y	page 4, line 14 - line 20 page 5, line 21 -page 6, line 10 page 29, line 4 -page 30, line 2 page 35, line 12 - line 18	56
X	WO 94 06920 A (MEDICAL RES COUNCIL ;RUSSELL STEPHEN JAMES (GB); HAWKINS ROBERT ED) 31 March 1994 (1994-03-31) page 19, last paragraph	55,57
A	GALL J. ET AL.: "ADENOVIRUS TYPE 5 AND 7 CAPSID CHIMERA: FIBER REPLACEMENT ALTERS RECEPTOR TROPISM WITHOUT AFFECTING PRIMARY IMMUNE NEUTRALIZATION EPITOPES" JOURNAL OF VIROLOGY, vol. 70, no. 4, 1 April 1996 (1996-04-01), pages 2116-2123, XP000653519 ISSN: 0022-538X the whole document	16-25
A	LYU Y. L. ET ALW: "Inversion/dimerization of plasmids mediated by inverted repeats." JOURNAL OF MOLECULAR BIOLOGY, vol. 285, no. 4, 29 January 1999 (1999-01-29), pages 1485-1501, XP002152918 ISSN: 0022-2836 cited in the application the whole document	1-58

-/--

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/15442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	STEINWAERDER D. S. ET AL.: "Generation of adenovirus vectors devoid of all viral genes by recombination between inverted repeats." JOURNAL OF VIROLOGY, vol. 73, no. 11, November 1999 (1999-11), pages 9303-9313, XP002152919 ISSN: 0022-538X the whole document ---	1-58
P,Y	LIEBER A. ET AL.: "Integrating adenovirus-adeno-associated virus hybrid vectors devoid of all viral genes." JOURNAL OF VIROLOGY, vol. 73, no. 11, November 1999 (1999-11), pages 9314-9324, XP002152920 ISSN: 0022-538X the whole document -----	1-58

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Interr nal Application No

PCT/US 00/15442

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 5856152	A	05-01-1999	AU	695811	B	20-08-1998
			AU	4405596	A	23-05-1996
			CA	2203808	A	09-05-1996
			EP	1046711	A	25-10-2000
			EP	0797678	A	01-10-1997
			JP	10507928	T	04-08-1998
			WO	9613598	A	09-05-1996
			US	5871982	A	16-02-1999
WO 9854346	A	03-12-1998	AU	7604998	A	30-12-1998
			BR	9809173	A	01-08-2000
			EP	0988390	A	29-03-2000
			PL	337130	A	31-07-2000
WO 9738723	A	23-10-1997	AU	2678097	A	07-11-1997
			CA	2251738	A	23-10-1997
			EP	0927044	A	07-07-1999
WO 9406920	A	31-03-1994	AU	689755	B	09-04-1998
			AU	4827893	A	12-04-1994
			CA	2145063	A	31-03-1994
			EP	0670905	A	13-09-1995
			JP	8504091	T	07-05-1996
			US	5723287	A	03-03-1998

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 December 2000 (07.12.2000)

PCT

(10) International Publication Number
WO 00/73478 A3

(51) International Patent Classification[7]: C12N 15/861, 15/864, 15/10, A61K 48/00

(21) International Application Number: PCT/US00/15442

(22) International Filing Date: 1 June 2000 (01.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/137,213 1 June 1999 (01.06.1999) US
60/161,097 22 October 1999 (22.10.1999) US

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF WASHINGTON [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105-4631 (US).

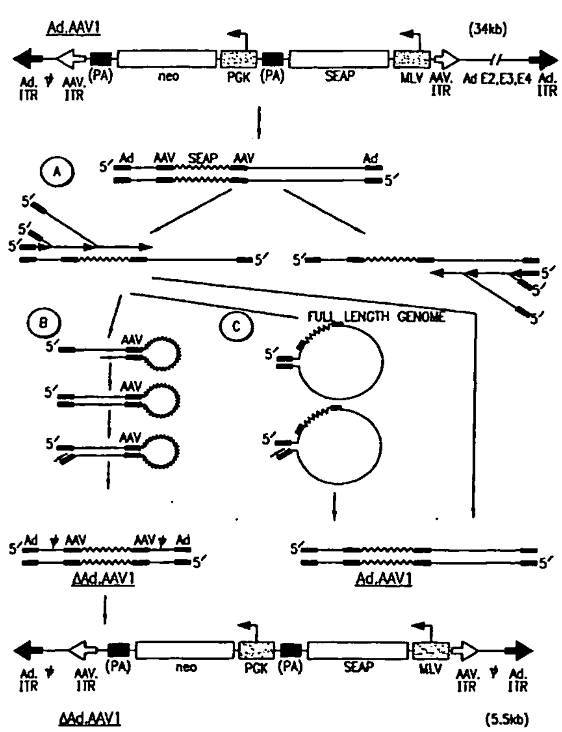
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LIEBER, André [DE/US]; 7251 29th Avenue, Seattle, WA 98115 (US). SHAYAKHMETOV, Dmitry [RU/US]; 6500 25th Avenue, Seattle, WA 98115 (US). FARRAR, Denise [US/US]; 1324 W. Emerson Street, Seattle, WA 98119 (US). PAPAYANNOPOULOU, Thalia [US/US]; 702 35th Avenue, Seattle, WA 98122 (US).

(74) Agent: ADRIANO, Sarah, B.; Mandel & Adriano, Suite 60, 35 No. Arroyo Parkway, Pasadena, CA 91103 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID,

[Continued on next page]

(54) Title: RECOMBINANT ADENOVIRAL VECTORS EXPRESSING CHIMERIC FIBER PROTEINS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION



(57) Abstract: The present invention provides for chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_v\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

WO 00/73478 A3

IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report: 5 July 2001

(48) Date of publication of this corrected version: 24 January 2002

(15) Information about Correction:
see PCT Gazette No. 04/2002 of 24 January 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

RECOMBINANT ADENOVIRAL VECTORS EXPRESSING CHIMERIC FIBER PROTEINS FOR CELL SPECIFIC INFECTION AND GENOME INTEGRATION

FIBER PROTEINS

This invention was made, at least in part, with funding from the National Institutes of Health (Grant Nos. R01 CA 80192-01 and R21 DK 55590-01). Accordingly, the United States Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to the field of gene therapy, and in particular, to novel adenovirus (Ad) vectors that selectively infect cells for gene therapy, and to Ad vectors containing modifications of the fiber protein to allow retargeting of any adenovirus serotype.

BACKGROUND OF THE INVENTION

Gene transfer vectors require the efficient transduction of target cells, stable association with the host genome, and adequate transgene expression in the appropriate target cell, without associated toxic or immunological side effects,. Currently available viral vector systems, including recombinant retroviruses, adenoviruses and adeno-associated viruses, are not suitable for efficient gene transfer into many cell types. Retroviral vectors require cell division for stable integration. Recombinant adenoviruses are not able to infect many cell types important for gene therapy, including hematopoietic stem cells, monocytes, T- and B-lymphocytes. Moreover, recombinant adeno-associated vectors (AAV) integrate with a low frequency.

First generation adenoviruses have a number of properties that make them an attractive vehicle for gene transfer (Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205). These include the ability to produce purified virus at high titers in concert with highly efficient gene transfer of up to 8 kb long expression cassettes into a large variety of cell

types *in vivo,* including non-dividing cells. Limitations of first generation adenoviruses include the development of immune responses to expressed viral proteins resulting in toxicity and virus clearance. The episomal status of adenoviral DNA within transduced cells is another limitation of first generation Ad vectors. Stable integration of adenovirus DNA into the host genome is reported only for wild-type forms of specific subtypes and appears not to occur in a detectable manner with E1/E3-deleted Ad 5 (adenovirus serotype 5) vectors widely used for gene transfer *in vitro* and *in vivo* [Hitt, M.M. et al. 1997 *Advances in Pharmacology* 40:137-205].

Recombinant AAV vectors (rAAV) integrate with a low frequency (about 1 out of 20,000 genomes) randomly as cocatemers into the host genome (Rutledge, E.A.; Russel, D.W. 1997 *J. Virology,* 71, 8429-8436). The presence of two AAV inverted terminal repeats (ITRs) and as yet unknown host cellular factors seem to be the only requirement for vector integration (Xiao, X., et al, 1997, *J. Virology,* 71, 941-948; Balague, C., et al. 1997, *J. Virology,* 71, 3299-3306; Yang, C.C. 1997, *J. Virology,* 71, 9231-9247). In the presence of the large AAV Rep proteins, AAV integrates preferentially into a specific site at human chromosome 19, called AAVS1 (Berns, K.I., 1996, *Fields Virology,* Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2173-2220). The AAV capsid is formed by three coat proteins (VP1-3), which interact with specific heparin sulfates on the cell surface and probably with specific receptor(s). However, many cell types, including hematopoietic stem cells, lack these structures so that rAAV vectors based on AAV2 cannot infect or transduce these cells (Malik P. et al., 1997, *J. Virology,* 71, 1776-1783; Quing, K.Y., et al. 1998, *J. Virology,* 72, 1593-1599). Other disadvantages of rAAV vectors include the limited insert size (4.5-5kb) that can be accommodated in rAAV vectors lacking all viral genes and low transducing titers of rAAV preparations.

Adenovirus infection is initiated by attaching to the cell surface of Ad 5 via its fiber protein (for a review, see Shenk, T. 1996 *Fields Virology, Vol. 2,* Fields, B.N. et al. (ed) Vol. 2, Lippincott-Raven, Philadelphia, PA, 2111-2148). The distal, C-terminal domain of the trimeric fiber molecule terminates in a knob, which binds to a specific cellular

receptor identified recently as the coxackie-adenovirus receptor (CAR) (Bergelson, J.M. et al. *Science*, 275, 1320-1323). After binding, in an event independent of virus attachment, Arg-Gly-Asp (RGD) motifs in the penton base interact with cellular integrins of the $\alpha3$ and $\beta5$ types. This interaction triggers cellular internalization whereby the virion achieves localization within the endosome. The endosomal membrane is lysed in a process mediated by the penton base, releasing the contents of the endosome to the cytoplasm. During these processes, the virion is gradually uncoated and the adenoviral DNA is transported to the nucleus where replication takes place. The terminal protein, which is covalently attached to the viral genome and the core protein V that is localized on the surface of the cores have nuclear localization signals (NLSs) (van der Vliet, B. 1995, *The Molecular Repertoir of Adenoviruses, Vol. 2*, Doerfler, W. and Boehm, P.(ed.), Springer Verlag, Berlin, 1-31). These NLSs play a crucial role in directing the adenoviral genome to the nucleus and probably represent the structural elements which allow adenovirus to transduce non-dividing cells. When the double-stranded, linear DNA reaches the nucleus, it binds to the nuclear matrix through its terminal protein.

Since the cell types that can be infected with Ad5 or Ad2 vectors are restricted by the presence of CAR and specific integrins, attempts have been made to widen the tropism of Ad vectors. Genetic modification of adenovirus coat proteins to target novel cell surface receptors have been reported for the fiber (Krasnykh, V. et al. 1998 *J. Virology*, 72, 1844-1852, Krasnykh, V. et al. 1996 *J. Virology*, 70, 6839-6846, Stevenson, S.D., et al. 1997, *J. Virology*, 71, 4782-4790), penton base (Wickham, T.J., et al. 1996, *J. Virology*, 70, 6831-6838; Wickham, T.J., et al. 1995, *Gene Therapy*, 69, 750-756), and hexon proteins (Crompton, J., et al. 1994, *J. Gen. Virol.* 75, 133-139). The most promising modification seems to be the functional modification of the fiber protein or more specifically of the fiber knob as the moiety, which mediates the primary attachment. Two groups have reported the generation of fibers consisting of the Ad5 tail/shaft and the knob domain of Ad3 (Krasnykh, V. et al. 1996 *supra*, Stevenson, S.D., et al. 1997, *supra*). Recently, recombinant adenoviruses with fibers containing C-terminal poly-lysine, gastrin-releasing peptide, somatostatin, E-selectin-binding peptide, or oligo-Histidines were produced in order to change the native tropism of Ad5. Krasnikh et al. found (Krasnykh, V. et al.

1998 *supra*) that heterologous peptide ligands could be inserted into the H1 loop of the fiber knob domain without affecting the biological function of the fiber. Based on studies with other Ad serotypes, it appears that the length of the fiber shaft is a critical element, determining the efficiency of interaction with cell surface integrins and the internalization process. Thus far, there is no reported data demonstrating successful retargeting of Ad5 vectors for a specific cell type.

Therefore, there is a present need for an improved adenovirus vector which can be targeted efficiently to a variety of cell types and tissues and remain stably integrated in the host genome with minimal antigenicity to the host. The present invention discloses novel chimeric adenoviral (Ad) Ad-AAV vectors, which express a modified fiber protein on their capsid, for specifically targeting the vector. Methods of making, uses and advantages of these vectors are described. In addition, the alteration described for the knob and shaft domains of the fiber protein provide a novel approach to retarget any adenovirus serotype for cell specific infection.

SUMMARY OF THE INVENTION

The present invention provides for novel chimeric Ad- vectors carrying transgene, or portions of transgenes for stable and efficient gene transfer into diverse cell types or tissues in a CAR- and/or $\alpha_\upsilon\beta_{3/5}$- independent manner. Also provided are methods for producing such vectors and the use thereof for gene therapy to target a specific cell type or tissue.

The recombinant adenovirus vectors of the invention (Example I) provide a novel design that allows for the easy production and delivery of a "gutless" adenoviral vector with the added advantage of stable integration of the transgene into the host genome of different cell type. The adenoviral vector described is devoid of all adenoviral sequences except for the 5' and 3' cis elements necessary for replication and virion encapsidation. The adenovirus-associated virus sequences of the invention comprising the 5' (right) and 3' (left) inverted terminal repeats (ITRs) flank the transgene gene cassette such that they

direct homologous recombination during viral replication and viral integration into the host genome. In one embodiment AAV-ITR flanking sequences are used. The vector also contains a selected transgene(s) operably linked to a selected regulatory element and a polydenylation stop signal, which is in turn flanked by the flanking sequences described above. The selected transgene(s)can be linked under the same regulatory elements or under separate regulatory elements in the same orientation or in opposite orientations with respect to each other. The selected transgene(s) are any gene or genes which are expressed in a host cell or tissue for therapeutic, reporter or selection purposes. This vector is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host genome. Also provided is a method to improve the integration frequency and site specific integration by incorporating an AAV rep protein into the recombinant hybrid vector.

The invention also provides chimeric fiber proteins (Example II), which includes naturally occurring fiber proteins in which a portion or portions of the sequence are modified to alter cell or tissue specificity of infection. Altered fiber protein sequences can include fiber protein domains (the knob domain, the shaft domain, and the tail domain) from other or the same adenovirus serotypes or from randomly selected peptides. A chimeric fiber protein can be entirely composed of non-naturally occurring sequences. The invention further relates to nucleic acid sequences encoding the chimeric fiber proteins. These nucleic acid sequences can be naturally occurring, a mixture of naturally occurring and non-naturally occurring sequences, or entirely non-naturally occurring sequences.

The heterologous fiber protein sequences described herein can be inserted into any adenovirus based vector which contains a capsid, rendering the virus capable of specifically infecting a given cell or tissue. Adenoviral vectors having such a heterologous fiber sequence can be used to direct gene transfer into desired cells. For stable integration of the transgene cassette into the host gemone, the chimeric Ad-AAV vector described in the invention is the preferred vector of use.

The invention also includes a library of adenoviruses displaying random peptides in their fiber knobs can be used as ligands to screen for an adenovirus variant with tropism to a particular cell type *in vitro* and *in vivo*.

The chimeric Ad- vectors described herein include the Ad.AAV genome with a modified fiber protein expressed on its capsid. These chimeric vectors are designed to infect a wide variety of cells, in particular, the cells which can only be poorly transduced by the commonly used retroviral, AAV and adenoviral vectors. These cells include, but not limited to, hematopoietic stem cells, lung epithelial cells, dendritic cells, lymphoblastoid cells, and endothelial cells. Hematopoietic stem cells such as CD34+ cells can be targeted for gene therapy of sickle cell anemia and thalasemia using the vector described herein. The chimeric Ad-AAV vector capable of transducing genes into endothelial cells can be used in gene therapy for vascular diseases such as atherosclerosis or restinosis after coronary artery surgery.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-1C display a proposed mechanism for forming of ΔAd.AAV1 genome.

Figs. 2A and 2B show electron photomicrographs of hybrid virus particles: Fig. 2A shows Ad.AAV1 and Fig. 2B shows ΔAd.AAV1.

Fig. 3 illustrates analysis of ΔAd.AAV1 genomes after transduction of SKHep1 cells. Pulse field gel eletrophoresis (PFGE). $1x10^6$ control Sk Hep1 cells (SKHep1) (lanes 1-3, 5, 9). SKHep1 cells from G418 resistant pools (ΔAd.AAV1) (infected with ΔAd.AAV1 and selected for 4 weeks) (lanes 6-8, 10-12), or SKHep1 cells collected at 3 days after infection with 2000 genomes Ad.AAV1 (Ad) lanes 4, 13) are sealed in agarose plaques, lysed *in situ* and subjected to PFGE with or without prior digestion with restriction endonucleases. Southern Blot is performed with a SEAP specific probe. U = undigested, P = digested with PI- Sce1, I = I-CeuI, E = EcoREI.

Figs. 4A and 4B show response of K562 and CD34+ cells respectively after infection with ΔAd.AAVBG. Cells are incubated for 6 hours with virus under agitation. At day 3 after infection, transduction frequency is calculated based on the number of X-Gal positive cells. Viability is tested by trypan blue exclusion. $N = 3$, $SEM < 10\%$.

Fig. 5 shows Rep expression in SKHep1 and 293 cells after plasmid transfection. 5×10^5 cells are transfected with pAAV/Ad, pRSVrep, or pPGKrep by Ca-phosphate co-precipitation. Three days after transfection, cells are harvested. Lysates are separated on a 10% PA gel, followed by Western Blot with Rep specific antibodies (03-65169), American Research Products), and developed with ECL (Amersham).

Fig. 6 shows detection of vector integration into AAVS1 by PFGE.

Fig. 7 shows strategy for creating an ΔAd.AAV hybrid vector capable of site-specific integration. Arrows indicate promoters, (PA) = polyadenylation signal. Ψ = adenoviral packaging signal.

Fig. 8A-8B shows vectors for transduction studies with SNori as expression unit and analysis of vector integration on genomic DNA from a small cell number. Analgous vector sets can be generated with β-galactosidase (BG) or green fluorescence protein (GFP) as reporter genes.

Fig. 9 shows strategy for substituting the Ad5 fiber sequence by the heterologous fiber X genes using recombination in *E. coli.*

Fig. 10 shows the expression of CAR and α_v-integrins on test cells. For flow cytometry analysis, HeLa, CHO, K562, and CD34+ cells were incubated with monoclonal anti-CAR (RmcB, 1:400 dilution) or anti-α_v-integrin antibodies (L230, 1:30 dilution). As a negative control, cells were incubated with an irrelevant mouse monoclonal antibody (anti-BrdU, 1:100 dilution). The binding of primary antibodies was developed with anti-mouse IgG-

FITC labeled conjugates (1:100 dilution). Data shown represent the average results of quadruplicate analyses performed on 10^4 cells.

Fig. 11 shows the electron microscopy of adenovirus particles. Purified particles from Ad5, 9, and 35 were negative contrast stained and analyzed at a magnification of 85,000x. Defective particles are highlighted by arrows.

Fig. 12 shows the analysis of attachment and internalization of different serotypes to CHO, HeLa, K562, and CD34+ cells. Equal amounts of [^{3}H]-thymidine-labeled virions of Ads 3, 4, 5, 9, 35, and 41 (measured by OD_{260}, and equivalent to an MOI of 400 pfu per cell for Ad5) were incubated for one hour on ice as described in Materials and Methods. Cells were then washed, and the number of labeled virions bound per cell was determined. For internalization studies, viruses were first allowed to attach to cells for 1 h on ice. Then, unbound viral particles were washed out. Cells were then incubated at 37°C for 30 min followed by treatment with trypsin-EDTA and washing to remove uninternalized viral particles. The data were obtained from two to four independent experiments performed in triplicate. Note the different scale on the Y-axes for CD34+ cells.

Figs. 13A-13C show attachment and internalization of different adenovirus serotypes to Hela, CHO and 293 cells respectively.

Figs. 14A and 14B show attachment and internalization of different adenovirus serotypes to CD34+ and K-562 cells respectively.

Fig. 15A-15C shows the analysis of viral replication in K562 and CD34+ cells by Southern blot analysis of methylated viral DNA. Replication studies were performed with 1×10^5 K562 cells (A) or CD34+ cells (B), infected with methylated Ad5, Ad9 or Ad35. The lane labeled as "load" represents DNA that was extracted form the media/cell mixture immediately after adding the indicated viral dose to cells. The intensities of bands corresponding to methylated and un-methylated viral DNA indicate that ~85% of

the input virus was methylated. To quantify adsorption and internalization, DNA analysis was performed after prior incubation of virus with cells at 0^0C (adsorption) or 37^0C (internalization). For dose dependent replication studies, the indicated viral dose (expressed as the number of genomes) was added to the cells, and cellular genomic DNA together with viral DNA was extracted 16 hours or 36 hours post-infection for K562 and CD34+cells, respectively. Identical amounts of sample DNA were analyzed by Southern blot. For quantification purposes, Ad9 replication was analyzed together with Ad5 using an Ad5/9 chimeric probe that hybridizes with DNA of both serotypes (C). The analysis of Ad5 versus Ad35 replication was performed with the corresponding Ad5/35 chimeric probe. Since separate hybridizations with both Ad5/35 and Ad5/9 probes gave identical signal intensities for Ad5 DNA only one panel is shown for Ad5 replication in test cells. To produce distinguishable fragments specific for the methylated or non-methylated status of viral genomes, Ad5 DNA was digested with *Xho* I, while Ad9 and Ad35 DNA was digested with *Xho* I and *Hind* III. The bands specific for methylated (not-replicated) viral DNA were ~12kb for Ad9, 35kb for Ad5, and ~12kb for Ad35. The fragments specific for non-methylated DNA were 5.8kb for Ad9, 6.1kb for Ad5, and 9.5kb for Ad35. Chimeric Ad5/9 and Ad5/35 DNA fragments (1.8kb) were used as quantification standards and applied onto gel together with digested viral/cellular DNA (shown on the left part of the figures).

Fig. 16A-16B shows the structure of Ad5GFP and chimeric Ad5GFP/F35 vectors. A) Schematic diagram of the original E1/E3 deleted Ad5-based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.coli. *Kpn* I (K) and *Hind* III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes. A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The

Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal. The region of Ad5GFP/F35 encoding for chimeric fiber was completely sequenced with Ad5 specific primers (see Material and Methods). B) Restriction analysis of viral genomes. Viral DNA was isolated from purified Ad5GFP and Ad5GFP/F35 particles as described elsewhere. One microgram of DNA was digested with *Hin*d III or *Kpn* I and separated in ethidium bromide stained agarose gels (left panel) which were subsequently blotted and analyzed by Southern blot with an Ad5 E4 specific probe (nt 32,7775-33,651) (right panel). Specific patterns, designating the correct structure for both viral vectors were detected. The *Hin*d III fragments specific for Ad5GFP and Ad5GFP/F35 were 2.9kb and 4.9kb, respectively. The *Kpn* I fragment that confirmed the correct Ad5GFP/F35 structure was 1.6kb compared to a 7.6kb Ad5GFP fragment. M - 1kb ladder (Gibco-BRL, Grand Island, NY).

Fig. 17 shows the generation of ΔAd.AAV genenomes by recombination between inverted homology regions. Recombination between two inverted repeats (IR) present in one Ad.AAV vector. The first-generation Ad.AAV vector (~34kb) contains two 1.2kb inverted homology elements flanking the transgene cassette. One AAV-ITR is inserted between the Ad packaging signal (ψ) and the left IR. During Ad replication, recombination between the Irs mediates the formation of the ΔAd.AAV genomes with the transgene flanked by Irs, AAVITRs, Ad packaging signals, and Ad ITRs. These genomes are efficiently packaged into Ad capsids.

Fig. 18 shows the structure of Ad5/11, Ad5/35. Schematic diagram of the original E1/E3 deleted Ad50based vector with GFP-expression cassette inserted into the E3 region (Ad5GFP) and the chimeric vector Ad5GFP/F35 containing the Ad5/35 fiber gene. The 2.2kb Ad5 fiber gene was replaced by a 0.9kb chimeric fiber gene encoding for the short shaft and knob of Ad35 by a technique that involved PCR-cloning and recombination in E.col. Kpn I (K) and Hind III (H) sites localized within or around the fiber genes are indicated. The lower panel shows the detailed structure of the chimeric fiber region. The Ad5 fiber tail [amino acids (aa): 1-44] were joined in frame to the Ad35 fiber shaft starting from its first two amino acids (GV), which are conserved among many serotypes.

A conserved stretch of amino acids TLWT marks the boundary between the last β-sheet of Ad35 shaft and the globular knob. The Ad35 fiber chain termination codon is followed by the Ad5 fiber poly-adenylation signal.

Fig. 19 shows the cross-competition for attachment and internalization of labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled viruses, and with anti-CAR or anti-α_v-integrins Mab. (A) For attachment studies, 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled competitor virus at 4°C for 1 h. Then, equal amounts of [^{3}H]-labeled viruses, at a dose equivalent to an MOI of 100 pfu per cell determined for Ad5GFP, were added to cells followed by incubation at 4°C for 1 h. Cells were then washed with ice-cold PBS, pelleted and the percentage of attached virus (cell-associated counts per minute) was determined. For analysis of cross-competition for internalization, cells were pre-incubated with a 100-fold excess of competitor virus at 37°C for 30 min before labeled virus was added. After an additional incubation at 37°C for 30 min, cells were treated with trypsin-EDTA for 5 min at 37°C, washed with ice-cold PBS, pelleted, and the percentage of internalized virus was determined. For controls, cells were incubated with labeled viruses without any competitors. Preliminary experiments had shown that the conditions chosen for competition studies allowed for saturation in attachment/internalization on K562 cells for all unlabeled competitors. (B) 10^5 K562 cells were pre-incubated for 1 hour at 4°C with anti-CAR MAb (RmcB, diluted 1:100) or with anti-α_v-integrin MAb (L230, diluted 1:30), followed by incubation with labeled viruses according to the protocols for attachment or for internalization as described above. For each particular serotype, the percentage of attached/internalized virus was compared to the control settings, where cells were preincubated under the same conditions with a 1:100 dilution of an irrelevant antibody (anti-BrdU Mab) before addition of the labeled virus. Note that the specific competitors but not the corresponding controls significantly inhibited Ad5 internalization to a degree that is in agreement with published data (59). N>/=4

Fig. 20. Cross-competition for attachment and internalization of [^{3}H]-labeled Ad5GFP, Ad35, and chimeric Ad5GFP/F35 virions with unlabeled Ad3 virus (A), and of [^{3}H]-

labeled Ad3 virions with unlabeled viruses (B). 10^5 K562 cells were pre-incubated with a 100-fold excess of unlabeled viral particles according to attachment or internalization protocols described for Fig.6. Equal amounts of [^{3}H]-labeled Ad5GFP, Ad5GFP/F35, or Ad35 (A) or [^{3}H]-labeled Ad3 (B) were added to cells at a dose equivalent to an MOI of 100 pfu per cell for Ad5GFP. In control settings, cells were incubated with labeled viruses without any competitors. N=4.

Fig. 21 shows the transduction of CD34+, K562, and HeLa cells with Ad5GFP and chimeric Ad5GFP/F35 vectors. 1×10^5 cells were infected with different MOIs (pfu/cell) of viruses in 100 μl of media for 6 hours at 37°C. Virus containing media was then removed, and the cells were resuspended in fresh media followed by incubation for 18 h at 37°C. The percentage of GFP expressing cells was determined by flow cytometry. N=3

Fig. 22 shows the distribution of GFP-positive cells in subpopulations of human CD34+ cells expressing CAR or α_v-integrins. 1×10^5 CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu/cell as described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CAR (1:100 final dilution) or anti-α_v-integrin (1:30 final dilution) primary MAbs for 1 h at 37°C. Binding of primary antibodies was developed with anti-mouse IgG-PE labeled secondary MAbs (1:100 final dilution) at 4°C for 30 min. For each variant, 10^4 cells were analyzed by flow-cytometry. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The data shown were representative for three independent experiments.

Fig. 23A-23B shows the distribution of GFP-positive cells in a subpopulation of human CD34+ cells, expressing CD34 and CD117 (c-kit). (A) Co-localization of GFP expression with CD34 or CD117: CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell under the conditions described for Fig.8. Twenty-four hours after infection, cells were incubated with anti-CD34 PE-conjugated MAbs (final dilution 1:2) or with anti-CD117 PE-conjugated MAbs (final dilution 1:5)

for 30 min on ice, and 10^4 cells per variant were subjected to two-color flow cytometry analysis. For negative control staining, no antibodies were added to the cells before analysis. The mock infection variants represent cells incubated with virus dilution buffer only. The quadrant borders were set based on the background signals obtained with both the GFP- and PE-matched negative controls. The percentages of stained cells found in each quadrant are indicated. The experiment was performed two times in triplicates, and typically obtained results are shown. The SEM was less than 10% of the statistical average. (B) Transduction of CD34+/CD117+ cells with Ad5GFP and chimeric Ad5GFP/F35 virus vectors: CD34+ cells, cultured overnight before staining in media without SCF, were incubated with PE-labeled anti-CD117 MAb for 30 min on ice. The fraction of CD117-positive cells was sorted by FACS. More than 97% of sorted cells were positive for CD117. $1x10^5$ CD117+/CD34+ cells were infected with Ad5GFP or Ad5GFP/F35 at an MOI of 200 pfu per cell, as for Fig.8. Twenty-four hours post infection, the percentage of GFP positive was determined by flow cytometry. For mock infection, CD117+/CD34+ cells were incubated with virus dilution buffer only. The infections were done in triplicates, and the average percentage of GFP-expressing cells is indicated on the corresponding histogram. The SEM was less than 10% of the statistical average.

Fig. 24 shows the southern analysis of viral genomes in GFP-positive and GFP-negative fractions of CD34+ cells infected with the Ad5GFP and chimeric Ad5GFP/F35 vectors. CD34+ cells were infected with viruses at an MOI of 100 as described for Fig.21. Twenty four-hours post infection, cells were sorted by FACS for GFP positive and GFP negative fractions. 10^5 cells from each fraction were used to isolate genomic DNA together with viral DNA. Before cell lysis, a rigorous treatment with trypsin and DNase followed by washing was performed to exclude that genomic DNA samples were contaminated by extracellular viral DNA. A) The upper panel shows the ethidium bromide stained 1% agarose gel before blotting demonstrating that similar amounts of genomic DNA were loaded. This amount corresponded to DNA isolated from ~25,000 GFP+ or GFP- cells. The lane labeled Aload@ represents viral DNA purified from Ad5GFP or Ad5GFP/F35 virions mixed with pBluescript plasmid DNA (Stratagene) as a carrier and applied on a

gel at the amount that was actually used to infect 25,000 cells. As a concentration standard, a serial dilution of Ad5GFP genomes was loaded on the gel (left side). For Southern analysis (lower panel), an 8kb-long HindIII fragment corresponding to the E2 region of Ad5 was used as a labeled probe. Hybridized filters were subjected to PhosphoImager analyis and then exposed to Kodak-X-OMAT film for 48 h at B70°C. The cellular/viral genomic DNA is indicated by an arrow. (B) To detect Ad5GFP genomes in transduced cells, PCR amplification followed by Southern blot hybridization was performed on the same samples that were used for quantitative Southern blot hybridization in (A). DNA purified from ~2,500 cells was subjected to PCR (95°CB1min, 53°C-1min, 72°CB 1min, 20 cycles with primers Ad5-F1 and Ad5-R1). One fifth of the PCR reaction was subjected to agarose gel electrophoresis (upper panel). A 0.9 kb-long DNA fragment, specific to the E4 region of Ad5 was detected for transduced Ad5GFP/F35 genomes. DNA then was blotted onto Nybond-N+ membrane and Southern blot hybridization (lower panel) with an Ad5 E4 specific DNA probe was performed. In addition to the 0.9kb DNA fragment, the PCR primers generated a smaller 0.5 kb-long fragment that also hybridized with with the E4 region probe.

Fig. 25 shows the role of fiber shaft length in Ad infection strategies. CAR binding (Ad5 and Ad9) variants and Ad35, which interacts with a non-CAR receptor were analyzed on CAR expressing cells (293, Y79) and K562 cells which do not express significant CAR amounts. All vectors contain a GFP expression cassette packaged into an Ad5 capsid with modified fibers.

Fig. 26 shows the tertiary structure of Ad5 knob: localization of CAR binding sites, H-I loop and G-H loop.

Fig. 27 shows the substitution of the G-H loop with heterologous peptides.

Fig. 28 shows the attachment and internalization of metabolically labeled serotypes with human cell lines.

Figs. 29A-29D shows the generation of Rep78 expressing Ad vectors by recombination between two vectors. (A) The same strategy outlined in Fig. 15 was employed for vectors with rep 78 as a transgene. The Ad5'rep vector also contained the ApoEhAAT promoter shielded by an HS-4 insulator. The region of homology between the two fragments of the rep78 gene was 658nt in length. The Rep78 ORF was deleted for the p5 promoter. The internal Rep 40/52 start codon (at position 993) was mutated to abolish production of the small Rep proteins. Furthermore, the splice site at nt 1905 was deleted eliminating production of Rep68. The individual expression of Rep 78 was demonstrated. (B) Formation of ΔAd.rep78 genomes. The expected 5.8kb ΔAdrep78 genome was only observed upon coninfection of both Ad5'rep and Ad3'rep into 293 celss as demonstrated by Southern. (C) Southern blot analysis for rescue of the recombinant AAV genome from plasmid DNA by Rep78 expressed from pCMVrep78 and ΔAd.rep78. The expected rescue product is 3.8kb (R-plasmid). (D) Southern blot analysis for rescue of the recombinant AAV genome from Ad.AAV viral vector genomes.

DETAILED DESCRIPTION OF THE INVENTION

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in in the present invention, the following words or phrases have the meanings specified.

The term vector includes, but is not limited to, plasmids, cosmids, phagemids, and artificial chromosomes. The vector sequence may be designated as the viral "base vector" sequence. The base vector sequence is dependent upon the particular type of virus and serotype that the base vector sequence was derived from. The base vector sequence may be linked to non-vector or transgene sequences (e.g., heterologous sequences).

The transgene sequences may include sequences that confer compatibility with prokaryote or eukaryote host cells, where compatibility relates to vector replication

within a host cell. Accordingly, the transgene sequence may be a replicon sequence that directs replication of the vector within the host cell, resulting in an autonomously replicating vector. Alternatively, the transgene sequence may permit vector replication that is dependent upon the host cell's replication machinery.

The base vector sequences may be a operatively linked to a transgene sequence that encodes a gene product, such as a polypeptide, rRNA, or tRNA. For example, the transgene may encode a polypeptide such as a viral capsid protein, or a viral fiber protein. The transgene may be derived from the same or different serotype as the base vector sequence.

Another example of a transgene includes a reporter gene that encodes a gene product that can be used as a selectable marker, such as drug resistance or a colorimetric marker. The reporter gene may encode a gene product which can be readily detected by, for example, a visual microscopic, immunochemical, or enzymatic assay. The preferred reporter gene encodes a gene product that can be detected by a non-destructive method that does not destroy the cell that expresses the reporter gene.

A therapeutic gene is another example of a transgene. A therapeutic gene encodes a gene product (e.g., polypeptide or RNA) which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or the tissue or the organ or the organism containing the host cell. The therapeutic benefit may result from modifying a functin of a gene in the host genome or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The base vector sequence may be linked to a transgene sequence that is an regulatory element, such as a promoters, enhancers, transcription termination signals, polyadenylation sequences. The regulatory element may direct expression of the transgene sequence that encodes a gene product by direct transcription or translation. The regulatory element may regulate the amount or timing of expression of the transgene

sequence. The regulatory element may direct expression of the transgene in certain host cells or tissues (e.g., host-specific or tissue-specific expression).

The base vector sequence may linked to a transgene sequence that permits the vector, to integrate into another nucleotide sequence. The integration sequence may direct integration of the whole vector or portions of the vector. The integration sequence may or may not be related to the base vector sequence. For example the integration and base vector sequences may be from the same or different viral serotype. The integration sequence may be inverted repeat sequences (ITRs) from adenovirus (Ad), adenovirus-associated virus (AAV), or HIV.

The base vector sequence may be linked to a transgene sequence that directs homologous recombination of the vector into the genome of a host cell. Such transgene sequences may or may not be from the same viral serotype as the base vector sequence.

The vector may be used to transport the heterologous sequence into a host cell or into a host cell's genome.

The vector may comprise multiple endonuclease restriction sites that enable convenient insertion of exogenous DNA sequences.

The term "hybrid vector" as used in the invention refers to a vector which comprises a nucleic acid sequence combined from two different viruses (e.g. Adenovirus and AAV).

"Chimeric vector" refers to a vector which contains nucleic acid sequences that are unnatural to the base vector (i.e. sequences not occurring naturally or sequences not in their natural background including heterologous sequences). A chimeric vector as used in the invention may also be a hybrid vector. An example of a chimeric vector is Ad.AAV expressing a modified fiber protein an its capsid.

The term "transduction" or "infection" refers to a method of introducing viral DNA within a virus particle into a host cell. The viral DNA herein is in the form of recombinant virus, which is generated by linking a segment of DNA of interest into the viral genome in such a way that the gene can be expressed as a functional protein.

The term "transfection" refers to a method of introducing a DNA fragment into a host cell.

The term "heterologous" as used herein means that a nucleic acid or peptide sequence is placed in a context that is not endogenous to the base adenovirus vector or to a transduced cell. For example, a peptide sequence can be transferred from a protein to another protein, the resulting protein is referred to herein as heterologous protein. A chimeric fiber protein, (e.g., a serotype 5 tail domain and a serotype 35 shaft and knob domain) is considered a "heterologous" to the Ad 5 vector. The term also includes nucleic acids (e.g. coding sequences) from one strain or serotype of adenovirus introduced into a different strain or serotype of adenovirus.

The term "regulatory elements" is intended to include promoters, enhancers, transcription termination signals, polyadenylation sequences, and other expression control sequences. Regulatory elements referred to in the invention include but are not limited to, those which direct expression of nucleic acid sequence only in certain host cells (e.g. tissue specific regulatory sequences).

The term "operably linked" indicates that a polynucleotide sequence (e.g. a coding sequence or gene) is linked to a regulatory element in such a way that the regulatory element sequence controls and regulates the transcription or translation or both of that polynucleotide sequence. The orientation of the regulatory element may vary (eg, be in reverse orientation with respect to the right ITR). The term also includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence and

production of the desired polypeptide or protein. Regulatory sequences can also include 3' sequences which ensure correct termination (eg. polyadenylation stop signal).

The term "gene therapy" used herein, refers to a method which introduces a segment of exogenous nucleic acid into a cell in such a way that it results in functional modification to the recipient cell by expression of the exogenous nucleic acid. The exogenous nucleic acid is typically therapeutic in that the expression of the encoded protein, polypeptide or RNA corrects cellular dysfunction due to a genetic error or more generally counteracts any undesirable functions which are associated with a genetic or acquired disease. The term "exogenous nucleic acid" refers to DNA or RNA sequences not normally expressed in the treated transformed cell. The term also refers to DNA and RNA sequences which are expressed in a treated transformed cell at a higher, lower or in an otherwise different pattern than in the untreated, nontransformed cell. This non-natural expression can also be termed heterologous expression.

A "gene therapy vector" refers to a vector used for gene therapy. i.e. to introduce the exogenous nucleic acid into a recipient or host cell. The exogenous nucleic acid may be transiently expressed or integrated and stably expressed in the recipient or host cell.

The term "plasmid" as used herein refers to any nucleic acid molecule which replicates independently of the host, maintains a high copy number, and which can be used as a cloning tool.

The term “parallel strand of DNA” and “anti-parallel strand of DNA” refers to as each of the strands of DNA of the double stranded adenovirus. The Figures diagram the location of certain nucleotides on the parallel strand of DNA. The anti-parallel strand of DNA refers to the other of the two strands of DNA which is not depicted in the Figures. The fibet protein is encoded on the anti-parallel strand of DNA. To simplify the vector diagrams, the fiber sequences are shown on the parallel strand even though the gene is located on the anti-parallel strand.

The term "reporter gene" refers to any nucleic acid sequence which encodes a polypeptide or protein which can be readily detected by, for example, a visual, microscopic, immunochemical or enzymatic assay. Preferred reporter genes are those that can be detected by a non-destructive method that does not destroy the treated, transformed cells or tissue.

The term "selection gene" used herein refers to any nucleic acid fragment which encodes a polypeptide or protein whose expression is used to mark a cell as a transformed cell by a given vector.

The term "therapeutic gene" refers herein to a DNA fragment encoding a functional polypeptide, protein or RNA, which when expressed in a host cell provides a therapeutic benefit or desired function to the host cell or to the organ or organism containing the host cell. The therapeutic benefit may result from modification of a function of a native gene in a host or from the additional function provided by the therapeutic protein, polypeptide or RNA.

The term "host tissue" or "host cell" as used herein, refers to a tissue or cell in which a therapeutic gene is to be expressed to modify its function.

It is well-known in the biological arts that certain amino acid substitutions may be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate, and isoleucine and valine, are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Volume 5, Supplement 3, Chapter 22, pp. 345-352, which is incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff et

al's frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources. Therefore, any obvious changes in the amino acid sequences (as described above) to the sequences of the invention are already contemplated.

Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups include but are not limited to: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, asparagine-glutamine, and aspartate-glutamate. Therefore, polypeptide substitution for "substantially similar" sequences (as described above) to the amino acid sequences described invention are already contemplated.

In order that the invention herein described may be more fully understood, the following description is set forth.

The present invention provides unique gene transfer vehicles which overcome many of the limitations of prior art vectors. The invention describes a first generation adenovirus vectors comprising left and right Ad ITRs, an Ad packaging sequence, a transgene cassette with regulatory elements, and a pair of cassette ITRs flanking the transgene cassette that direct predictable viral genomic rearrangements during viral replication as well as direct the integration of the transgene cassette into the host cell genome.

One predictable rearrangement that occurs during viral replication is the generation of a gutless adenovirus vector (also referred to herein as ΔAd) that comprises right and left Ad ITRs, an Ad packaging sequence, a transgene cassette flanked by cassette ITRs and the gutless vector is devoid of all other immunogenic viral genes.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the transgene cassette is directed by co-infection with an Ad vector expressing e.g., the rep 78 protein to achieve site-specific integration in the e.g., AAVS1 site on human chromosome 19.

The invention further describes a novel way of targeting these recombinant adenovirus vectors to selected cells by modifying the adenovirus fiber protein that is expressed on the capsid. Changes to both the fiber shaft and the fiber knob domain proved to successfully retarget the Ad vector to a desired cell type. In addition, the G-H loop within the fiber knob domain is identified as a novel site that affect the binding affinity and specificity of the recombinant adenovirus vector. Substitution of peptide sequences into the G-H loop retarget the gutless vector to a desired cell type.

An adenovirus display library has been generated that expresses random peptides within the G-H loop of the fiber protein. This type of a library is used as ligands to screen for adenovirus vectors that bind to desired cell types. One advantage of using an adenovirus display library versus a phage display library is that once adenovirus affinity to a desired cell is identified the targeted adenovirus vector is ready to accept a transgene cassette and can be used to generate a gutless adenovirus vector, for example for use in gene therapy.

The chimeric Ad vectors described below contain a modified fiber protein in the capsid of the adenovirus which renders the vector capable of infecting a desired cell types. Therefore, according to the invention, a gutless chimeric ΔAd-AAV vector can be generated to introduce any transgene(s) into any host cell or tissue which is normally refractory to most commonly used gene therapy viral vectors. In addition, the chimeric ΔAd.AAV vector of the invention, is devoid of adenoviral genes, and contains AAV ITR

sequences that flank the transgene cassette, which direct stable transgene integration in the host genome allowing long term expression of the transgene.

The transgene cassette described in the invention may carry a transgene which is either a reporter gene, a selectable gene for in vitro or in vivo selection of transduced cells, or a therapeutic gene. In one embodiment of the invention the reporter trangene can be but is not limited to, βgalactosidase. Many reporter genes are commonly used in the art, of which any could be carried as a transgene in the Ad.AAV vector of the invention. Other examples of reporter are genes are GFP and alkaline phosphatase.

The following describes an embodiment of the first generation Ad vectors of the invention having a wild-type capsid and a transgene cassette flanked by cassette ITR sequences; (b) fiber protein that is modified to retarget Ad vectors; and (c) the combination of both technologies that enables the production of chimeric ΔAd vector including a modified fiber protein expressed on the capsid which retargets the base vector to a desired cell type for infection and transgene integration.

A. Integrating Ad hybrid vectors of the invention:

It has been shown that inverted repeats (IRs) inserted into the E1 region of AdE1- vectors can mediate predictable genomic rearrangements resulting in a gutless vector genome devoid of all viral genes. A specific embodiment of such IR-mediated rearrangements is the Adeno-AAV, first generation hybrid adenovirus vector containing AAV inverted terminal repeats (ITR) flanking a transgene cassette. The AAV ITRs mediate the formation of a genome similar to that of the ΔAd.IR genome (Steinwaerder et al., 2000 Journal of Virology). ΔAd vectors devoid of all viral genes stably integrate and transduce cultured cells with efficiencies comparable to e.g. rAAV vectors. The Examples demonstrate by Southern blot analysis that the ΔAd vectors integrate randomly into the host genome.

The Ad vectors of the invention comprise a left Ad ITR, an adenovirus packaging sequence located 3' to the Ad ITR; a transgene cassette located 3' to the packaging

sequence comprising a polyadenylation signal, a transgene, and a heterologous promoter, and flanked by a pair of cassette ITRs. Adenoviral genes used for replication such as E1, E2, E3, E4 are located 3' to the right cassette ITR and a right Ad ITR is located 3' to the replication genes. The vectors of the invention are particularly suited to treat: genetic disorders, cancers, and infectious diseases (such as HIV, emboli, or malaria). Treatable genetic diseases such as hemophilia A and B; cystic fibrosis; muscular dystrophy, and $\alpha_1$antitrypsin disorder are ideal candidates for genetic disease that can be treated by vectors of the invention. A specific example of a therapeutic gene to combat a genetic disorder is gamma-globin to ameliorate sickle cell anemia.

To aid in the selection of transduced cellls and characterize the intergration site of the transgene cassette, an embodiment of the invention includes the addition of a sequence comprising a bacterial for the origin of replication, plus a selectable gene. An embodiment of this is an SNori sequence added to the transgene cassette. This allows the ΔAd to be expressed in human and bacterial cells, therefore allowing selection of the transduced cells and characterization of the integration site in the genome of transduced mammalian cells.

The potential for site-specific integration is an important characteristic of the novel Ad vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein in 293 cells to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

Integration of the transgene cassette contained in the adenoviral vectors into chromosomes may be associated with silencing (or blocking) of transgene expression. The silencing of transgenes can be overcome by adding insulator elements to the transgene cassette. For example, HS-4 insulator elements derived from the chicken-globin LCR can function in Ad vectors to shield heterologous promoters from adenoviral enhancers. HS-4 insulators or the *Drosophila* Gypsy gene can also be used to prevent silencing transgenes.

Another embodiment of the invention is to split the transgene cassette into two portions of the transgene each carried in a different recombinant adenoviral vector of the invention. Each portion of the same trangene has an overlapping region of homology. After infection with both vectors, each carrying the different but overlapping portion of the same transgene, homologous recombination event occurs resulting in the reconstitution of the complete transgene which is then expressed. This technique is used to produce hybrid adenoviral vectors that accommodate large inserts including, but not limited to a 13kb genomic hAAT gene or a 12kb γglobin LCRγglobin expression cassettes for ameliorating sickle cell anemia (or correcting γ-globin mutations). The formation of the hybrid ΔAd vector genomes, after recombination between two vectors, is more efficient if the overlapping region of homology within the transgene is longer.

An advantage of the present invention is a method to rapidly isolate pure gutless hybrid adenoviral vectors such as ΔAd.AAV or ΔAd.AAV^{fx} vectors. To minimize the contamination of ΔAd with first generation vectors (Ad vectors) a strategy is described in Example I H. It is anticipated that these approaches will yield the same titer of ΔAd vectors, however the contamination with full-length genome vectors will be less. This improved isolation of the vectors is extremely important to avoid toxic side effects after in vivo application.

B. <u>Tropism modified adenovirus vectors:</u>

The Ad vectors of the invention can be modified so that they target a host cell of interest. There are more than 50 human Ad serotypes (Appendix I), including variants with different tissue selectivity or tropism. It is accepted in the art that different Ad serotypes bind to different cellular receptors and use different entry mechanisms. Most recombinant adenovirus vectors use adenovirus serotype 5 as the base vector serotype 5 (Ad5) (Hitt, M.M., et all, 1997, Adv. in Pharmacology 40, 137-205). Ad5 infection is primarily mediated by its fiber protein binding to CAR and secondarily by its penton base protein binding to integrin. Due to the lack of CAR and/or integrin expression on many cell and tissue types, Ad5 mediated gene transfer is inefficient in a number of tissues which are important targets for gene therapy such as endothelia, smooth muscle, skin epithelia, differentiated airway epithelia, brain tissue, peripheral blood cells, or bone marrow. The following describes Ad5 vectors of the invention having a change in infectivity and tropism as a result of altering the fiber protein sequence.

The infectivity of different Ad serotypes is limited to a number of human cell lines. Infectivity studies revealed that Ad5 and Ad3 are particularly suitable for infecting and targeting endothelial or lymphoid cells, whereas Ad9, Ad11 and Ad35 efficiently infected human bone marrow cells. Therefore, the knob domain of the fiber protein of Ad9, Ad11 and Ad35 are excellent candidates for retargeting the Ad5 vector to human bone marrow cells. Other possible serotypes include Ad7.

In the modified fiber protein of the invention the fiber knob domain of the Ad5 fiber has been replaced with another Ad serotype fiber knob domain. An embodiment of the invention is the modified Ad5/35 fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising of a fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad35). The Ad5/35 chimeric fiber protein shows a broader spectrum of infection to a subset of CD34+ cells, including those with stem cell activity. The Ad5/11 chimeric fiber protein (a recombinant Ad5 vector expressing a modified fiber protein comprising the fiber tail domain of Ad5 and the fiber shaft and knob domains of Ad11) showed similar tropism.

In addition to the knob domain modifications, the invention describes the added advantage of modifying both the fiber shaft domain and the fiber knob domain to produce a shortened fiber protein. The length of the fiber shaft domain plays a key role in the host receptors used for viral vector entry into the host cell. To show this Ad5, Ad5/9, and Ad5/35 variants were constructed with long (22β-sheets) and short-shafted (7β-sheets)-shafted fibers. These analyses demonstrated that efficient viral infection involving CAR as the primary receptor for Ad5, Ad5/9 requires a long-shafted fiber protein, whereas the cell entry strategy of Ad5/35 (which binds to an still uncharacterized non-CAR receptor) does not depend on the shaft length (Fig. 3). The modification in both the fiber shaft domain length (between 5 > 10 β-sheets) and the fiber knob domain (from a different Adserotype than the base vector is a novel mode of altering Ad vector tropism.

To broaden the repertoire of cell types that Ad vectors can infect, a specific binding region, the G-H loop, within the knob domain has been newly identified herein to improve binding affinity and specificity. Alteration within this region will redirect the Ad vector to a desired cell type. For example, the invention describes the G-H loop sequence within the fiber protein knob domain, which can be replaced with heterologous peptide ligand sequences without affecting the functionally important tertiary structure of the Ad fiber knob domain, while changing the binding affinity and specificty of the vector (Figs. 6,7). This G-H loop region is exposed on the central part of the knob surface and may be strategically a better site for incorporation of heterologous ligands than the peripheral H-I loop (Krasnykh, V. et al., 1998, *J. Virol.*, 72:1844-52.) of the knob C-terminus (Michael, S. I., et al., 1995, *Gene Ther.*, 2:660-8., Wickham, T. J. et al., 1996, *Nat. Biotechnol.*, 14:1570-3.), which are the substitution sites used by others. Therefore, these G-H loop modifications within the fiber knob domain will allow the Ad vector to be redirected to infect a desired cell type, as long as the G-H loop ligand sequence binds to at least one surface protein on the desired cell type. Fig. 7 shows some possible substitutions. Example II J demonstrates that the virion tolerates the insertion of a cycling peptide (12 amino acids) with a constrained secondary structure that allows the exposure on the knob surface. A defined ligand (RGD) can be inserted into the G-H and the H-I loop of an

Ad5 capsid that is ablated for CAR, and integrin tropism. Infectivity studies show the potential advantage of this new insertion site.

Use of the Vectors of the Invention for "Gene Therapy"

The liver is the major organ for protein synthesis. Thereforean important goal of gene therapy is to target gene therapy vectors to the liver. To genetically correct many types of mutant proteins hepatocytes need to be infected with gene therapy vectors carrying a corrected transgene. Example II J describes a G-H loop substitution in the knob domain of the fiber protein with both RI and RII+ (of the malaria circumsporozite surface protein) in a short shafted fiber protein which directs the vector to have affinity and specificity to hepatocytes.

Example II K applies a similar protocol to alter the fiber knob domain in the G-H loop region with peptides that target the vector breast cancer cell lines (MDA-MB-435). These novel approaches to redirect vectors described in the invention allow lower doses of the gene therapy vectors to be administered with a higher safety profile.

In example II L a protocol for preparing an adenovirus display library is described that uses the fiber knob protein to display a library of random peptide sequences within the G-H loop. This library of adenoviruses with modified fiber proteins is screened for affinity and specificity for a desired cell type. There are two main advantages of using this adenovirus display library to screen for target peptides that allow binding to a desired cell type over a phage display library system. First, once a ligand peptide is identified that binds to the desired cell type it is already in the vector of choice for gene therapy delivery. The peptide does not need to be engineered into another vector, as is the case for the phage display library vectors. This reduces the steps required to identify a targeted fiber protein for a desired cell type. The second advantage of this method, is that the adenoviruses are able to display multiple copies of the modified fiber protein on their capsid. This allows for dimerization and trimerization of the fiber protein with the host cell receptor. The multimerization of fibers proteins is a realistic, in vivo interaction of

the trimeric fiber protein with the host cell receptor. In contrast, phage vectors can only display one fiber peptide sequence on their surface, which significantly limits the ability of interaction with host cell surface receptors.

C. A chimeric adenovirus vector with selective tropism:

The chimeric vectors of the invention combine two vectors: an Ad.ITR and a Ad. fx where fx describes a modified fiber protein. A first generation adenovirus vector of serotype 5 is the base vector that carries a transgene cassette flanked by heterologous ITRs. These specific inverted terminal repeat sequences, such as AAV ITRs direct stable integration of the transgene cassette into the host genome as well as control predictable genomic rearrangements that occur during viral replication. This vector can also carry a modified fiber gene (described in Examples II). During replication predictable genomic rearrangements occur which result in the generation of a gutless adenovirus vector (e.g. ΔAd.AAV^{fx}) which expresses the modified fiber protein on its capsid. The modified fiber protein allows the gutless vector to be targeted to a selected cell type. The targeted vector is a gutless adenovirus vector devoid of adenoviral genes which can integrate its transgene into the host genome. The transgene cassette can carry reporter, selectable, or therapeutic genes.

In one embodiment of the invention, the gutless targeted ΔAd.AAV^{fx} carries the reporter gene of $\tilde{\beta}$galactosidase. ΔAd.AAV^{fx} $^{-}$BG). For easy in vitro selection of human and bacterial cells that are transduced with the hybrid Ad vector, a bacterial sequence for the origin of replication can be added to the hybrid Ad vectors. An example of this is ΔAd.AAV^{fx} -Snori, in which a SNori sequence is added into the transgene cassette. This site allows for G418 selection on cells infected with ΔAd.AAV^{fx} –SNori. This in vitro selection provides a tool to analyze the site of transgene integration and the flanking chromosomal regions. Fluorescent in situ hybridization (FISH) is an alternative method to confirm vector integration.

An advantage of the ΔAd chimeric vector for gene transfer is the efficient and stable integration of a large transgene cassette up to e.g. 22kb which is significantly larger than the capacity of retroviral vectors . This is of particular interest for gene therapy. For example, to ameliorate sickle cell anemia ΔAd.AAV^{fx} $\bar{\gamma}$globin , an expression transgene cassette with the gamma-globin gene that targets and integrates, can be inserted into bone marrow stem cells for long term expression of the gamma-globin gene.

To achieve site-specific gene integration, rep78 protein is used for transgene integration into the AAVS1 site (described in Example 1 D). However, this may silence transgene expression. To prevent the integrated transgene from being silenced by host genomic elements (such as positional effects or downstream enhancers), LCRs or insulator elements are incorporated into the transgene cassette.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE I

NOVEL ADENOVIRAL VECTOR Ad.AAV

A. Integrating ΔAd.AAV hybrid vectors devoid of all adenoviral genes.

In vitro and *in vivo* studies with rAAV indicate that the only requirement for rAAV integration are the AAV ITRs and as yet unknown host cellular factors. It is thought that specific sequences or secondary structures present in AAV ITRs are prone to integration into host chromosomal DNA. In order to combine advantages of adenoviral vectors (high titer, high infectivity, large capacity) and the integration capability of AAV ITRs, AAV vector DNA with AAV ITRs flanking cassettes a secreted human placental alkaline phosphatase (SEAP) - neomycin phosphotransferase (neo) reporter gene cassette

(Alexander, I.E., et al. 1996, *Gene Therapy*, 7, 841-850) is incorporated into the E1-region of E1/E3 deleted adenoviral vectors (Ad.AAV1) (Figure 1, top).

METHODS

<u>*Production/Characterization of Viral Vectors*</u>

<u>*Plasmids*</u>:

The AAV1 vector cassette containing AAV ITRs and SEAP/neo expression units is obtained by AseI/ScaI digestion of the plasmid pALSAPSN (Alexander, I. E. et al, 1996, *Human Gene Therapy,* 7:841-50). The 4.4kb AAV vector fragment was cloned via NotI adapter linkers into pXJCL1 (Mirobix, Toronto, Canada) (pAd.AAV1). Another shuttle vector (pAd.AAV1-Δ2ITRs) lacking the AAV ITRs is generated by inserting the 3.7kb AflII/BsmI fragment of pALAPSN into pXJCL1. For pAd.AAV1Δ1ITR, a construct is used where a spontaneous deletion in the left AAV ITRs between the A and A' regions has occurred. To create a second hybrid vector (Ad.AAV2), the AAVSNori cassette developed by E. Rutledge is used. AAV vector DNA obtained is from pASNori (Rutledge, E. A., Russell, D.W. 1997. *Journal of Virology* 71:8429-8436) as a 3.4kb BsaI/ScaI fragment and inserted into the EcoRV site of pXCJL1. As it is generally known for AAV vector plasmids, the AAV ITRs are prone to rearrangements. To minimize deletions in these functional critical regions, all constructs for generation of hybrid vectors are assembled in low copy-number plasmids which are grown in E. coli Top10, JC811, or XL1 Bluecells (Stragene, La Jolla, Calif.). Furthermore, after each cloning step or large-scale plasmid amplification, both AAV ITRs are carefully mapped by restriction analysis with enzymes that cut inside or adjacent to the ITRs (BssHII, AhdI, SmaI, BglI, BsmI, AflII, and ScaI).

Adenoviruses:

First-generation viruses with the different transgene cassettes incorporated into the E1 region are generated by recombination of the pΔE1aSpla- or pXCJL1-derived shuttle plasmids and pJM17 (Microbix) in 293 cells as described earlier (Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791). For each virus, at least 20 plaques are picked, amplified, and analyzed by restriction digest. Viruses containing two AAV ITDRs tend to rearrange within the ITRs, with other adenoviral sequences, or with adenoviral sequences present in the 293 cell genome. Only plaques from viruses with intact ITRs are amplified, CsCl banded, and titered as described earlier (Kay, M. A., et al. 1995. *Hepatology* 21:815-819; Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960). All virus preparations tested are negative for RCA and bacterial endotoxin (Lieber, A., et al.1997. *Journal of Virology* 71:8798-8807). Virus is stored at –80°C in 10mM Tris-Cl, pH 7.5-1 mM $MgCl_2$-10% glycerol.

To generate ΔAd.AAV, 293 cells are infected with Ad.AAV1 at an multiplicity of infection (MOI) of 25 and harvested 40h after infection. Cells are lysed in PBS by 4 cycles of freeze/thawing. Lysates are centrifuged to remove cell debris and digested for 30 min at 37°C with 500 units/ml DNaseI and 200 μg/ml RNaseA in the presence of 10mM $MgCl_2$. 5ml of lysate is layered on a CsCl step gradient (0.5ml - 1.5g/cm^3, 2.5ml - 1.35g/cm^3, 4ml - 1.25g/cm^3) and ultracentrifuged for 2h at 35,000 rpm (rotor SW41). CsCl fractions are collected by puncturing the tube and are analyzed for viral DNA (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960; Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) or subjected to ultracentrifugation at 35,000 rpm for 18 hours in an equilibrium gradient with 1.32 g/cm^3 CsCl. The band containing the deleted viruses ΔAd.AAV is clearly separated (0.5cm distance) from other banded viral particles containing full-length ad.AAV genomes. ΔAd.AAV1 fractions are dialyzed against 10mM Tris-Cl, pH 7.5-1mM $MgCl_2$-10% glycerol and stored at –80°C. The genome titer of ΔAd.AAV1 preparations is determined based on quantitative Southern analysis of viral DNA purified from viral particles in comparison to different concentrations of a 4.4kb AseI/ScaI fragment of pALSAPSN according to a protocol described earlier (Lieber, A.,

et al., 1996, *J. of Virology*, 70, 8782-8791). In total, the production of $1x10^{13}$ genome-particles of ΔAd.AAV1 requires less than 3 hours of actual work.

Titers routinely obtained are in the range of $3\text{-}8x10^{12}$ genomes per ml. Assuming one genome is packaged per capsid, the genome titer equals the particle titer. The level of contaminating Ad.AAV1 is less than 0.1% as determined by Southern analysis, which is consistent with results obtained by plaque assay on 293 cells (fewer than 5 plaques per 10^6 total genomes). The primers used for sequencing the left and right ITR-vector-junction are

5'GGCGTTACTTAAGCTAGAGCTTATCTG, and
5'CTCTCTAGTTCTAGCCTCGATCTCAC.

The recombinant AAV virus stock containing the SEAP/neo cassette (AV2/ALSAPSN, [Alexander, I. E. et al, 1996, *Human Gene Therapy,* 7:841-50] used in these studies were obtained from Dusty Miller (FHCRC, Seattle). The stock was free of contaminating replication competent AAV (<50 particles/ml) and wildtype adenovirus (<100 particles/ml). The genome titer of the virus stock was obtained by quantitative Southern Blots as described by Russell et al. (Russell, D. et al. 1994 *Proc. Natl.Acad,Sci. USA* 91:8915-8919).

Electron Microscopy:

For examination of viral particles in the transmission electron microscopy studies, CsCl-purified virions are fixed with glutaraldehyde and stained with uranyl acetate as described previously (Lieber, A., et al. 1996. *Journal of Virology* 70:8944-8960).

RESULTS

During replication of these hybrid vectors in 293 cells, a 5.5kb genome (ΔAd.AAV1) is efficiently generated and packaged into adenovirus (Ad5) capsids. The ΔAd.AAV1

genome contains the left adenovirus ITR and the packaging signal followed by the AAV-vector cassette and a duplicate of the adenoviral packaging signal and ITR in reverse orientation (Figure 1, bottom). The hybrid vector is devoid of all viral genes, thus eliminating toxic effects and the elicitation of cellular immune responses. The spontaneous formation of the small hybrid vector genome ΔAd.AAV1 requires the presence of two intact AAV ITRs and does not occur with partly deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette.

Hybrid vectors containing different transgenes:

To construct a hybrid vector with a transgene that can be detected in situ the SEAP/neo expression unit in Ad.AAV1 is replaced by the *E. coli* β-galactosidase gene. This hybrid vector is named ΔAd.AAV1. During generation of the corresponding plasmid constructs the AAV ITR sequences tend to rearrange and abolish their functional properties. This problem can be circumvented by using low copy number plasmids as cloning vectors grown in bacteria strains depleted for all recombination proteins (e.g.JC811). Furthermore, the intactness of both AAV ITRs after each cloning step can be examined for characteristic endonuclease digestion. Recently, another hybrid vector ΔAd.AAV1Nori has been generated which contains the neo gene under the control of both the simian virus 40 (SV40) early promoter and the transposon 5 (Tn5) promoter for expression in human and bacterial cells, as well as the p15A bacterial replication origin with the direction of the leading strand DNA synthesis opposite that of neo gene transcription. Thus, SNori can be used for G418 selection of integrated vector in eukaryotic cells as well as for rescue of vector together with flanking host DNA after integration. The recovered plasmids can be propagated in *E. coli* under selection with kanamycin due to the bacterial origin and the neo gene. SNori containing vectors allow a rapid estimation of total integration events based on the number of G418 resistant colonies. Moreover, vector DNA together with flanking chromosomal DNA can be rescued as plasmids from single G418 resistant clones and can be used for sequencing to determine integration junctions. Both hybrid vectors are produced at a titer of about

$3x10^{12}$ genomes per ml. The ratio of genome titer to transducing particles for ΔAd.AAVBG is ~200:1 based on β-Gal expression.

DISCUSSION

ΔAd.AAV1 could spontaneously form during adenovirus replication. Another possible mechanism of ΔAd.AAV1 formation is based on the unique mechanism by which adenovirus replicates its genome (van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin) (see Figure 1). Ad DNA replication is initiated by the TP/pTP (terminal protein) that binds to specific sites within the ITRs on both ends of the linear genome and catalyzes, in complex with Ad pol, the binding of the 5' CTP, the first nucleotide of the daughter strand. DNA synthesis proceeds in a continuous fashion to the other end of the genome (Figure 1A). Only one of the DNA strands serves as template. One of the replication products is a single-stranded DNA that circularizes through annealing of its self-complementary ITRs. The resulting duplex "panhandle" has the same structure as the termini of the duplex viral genome that allows the binding of pTP and the initiation for synthesis of a complementary strand using the single-stranded "pandhandle" molecule as template (Figure 1C). In the case of Ad.AAV1, the Ad pol synthesizes the single strand of the adenoviral genome starting from the left Ad ITR until it reaches the second AAV ITR. During synthesis of the second AAV ITR a certain percentage of the single-stranded molecules form a loop hybridizing to the complementary region within the first AAV ITR that was replicated earlier, allowing Ad pol to use the same viral DNA strand to read back towards the left ITR (Figure 1B). The resulting "panhandle" structure can be resolved in a similar way as a full-length intermediate shown in Figure 1C, generating a double stranded, linear molecule with the above described structure that can be packaged into Ad virions. The ratio of viral DNA to protein concentration in purified ΔAd.AAV1 particles is comparable to that obtained from Ad.AAV1 particles. This indicates that despite the smaller size, only one ΔAd.AAV1 genome is packaged, resulting in particles with a lighter buoyant density (~1.32 g/cm^3). Electron microscopy demonstrates the icosahedral shape of ΔAd.AAV1 particles (Figure 2). Staining with uranyl acetate causes the central viral cores to appear

electron dense. ΔAd.AAV1 virions have only a spotted luminal dark staining as expected with only one 5.5kb genome being packaged per capsid.

B. *In vitro* ΔAd.AAV1 production:

Characteristics of deleted adeno-AAV vectors (ΔAd.AAV):

A number of experiments to clarify the mechanisms of ΔAd.AAV genome formation are carried out. Specifically, the presence of two intact AAV ITRs flanking a reporter gene cassette is required for the effective formation of ΔAd.AAV genomes. This process does not occur with partially deleted ITRs or oligo-dC and oligo-dG stretches flanking the expression cassette. Furthermore, in vitro transduction studies are performed with different genome titers of ΔAd.AAV1, Ad.AAV1, and Ad.AAV1-Δ2ITRs, (lacking the two AAV ITRs) which determine the number of G418 resistant colonies that formed after 4 weeks of selection (Table I).

ΔAd.AAV1 is routinely produced at a high titer (5×10^{12} genomes per ml with $>10^4$ produced genomes per 293 cell) and at a high purity with less than 0.1% contaminating full length Ad.AAV1 genomes by a technique normally used for amplification and purification of recombinant adenovirus.

In vitro transduction studies with hybrid vectors on CD34+ cells and erythroleukemia cells:

In order to test whether the hybrid vectors allow for gene transfer into cell types, that have to be targeted for sickle cell therapy, infection/transduction studies are performed using CD34+ enriched human bone marrow cells, derived from mobilized peripheral blood and the human erythroleukemia cell line K562 which express ε and γ globin genes.

METHODS

Cell Culture:

SKHep1 cells (HTB-52, American Type Culture Collection, Rockville, MD), an endothelial cell line derived from human liver [Heffelfinger, S.C., et al., 1992, *In vitro Cell Dev. Biol.* 28A, 136-4-142], are grown in high-glucose Dulbecco's modified Eagle medium with 10% fetal calf serum. SKHep1 cells are analyzed for integrated AAV provirus by Southern analysis of genomic DNA using the AAV1 wild type genome obtained from pAAV/Ad (Samulski, R. J., et al. 1989. *Journal of Virology* 63:3822-3928) (gift from David Russell, University of Washington) as a probe. No specific bands are detected in undigested genomic SKHep1 DNA or after digestion with HindIII. For viral infection, confluent cells are incubated with different viral doses for 2 hours, followed by intensive washing. For G418 selection, 24h after infection with ΔAd.AAV1, SKHep1 cells are trypsinized and plated at different dilutions under G418 selection (900μg/ml active compound, Boehringer-Mannheim, Germany). G418 containing culture medium is changed every 3 days. The number of colonies with $>10^4$ cells is counted after 4 weeks of selection and divided by the number of initially seeded cells. This ratio is used to express the integration frequency of ΔAd.AAV1. Single colones are obtained by limiting dilutions of infected cells in 96 well plates. Colonies are expanded to $1x10^6$ cells in the presence of G418. Immunofluorescence analysis for adenoviral proteins expressed in SKHep1 cells 3 days post-infection is performed as described earlier [Lieber, A., et al., 1996, *J. of Virology*, 70, 8782-8791].

RESULTS

293 cells are infected with the first generation vector Ad.AAV1. During replication of Ad.AAV1, the small ΔAd.AAV1 genome forms spontaneously and is packaged into adenovirus capsid. At 36 hours after infection cells are harvested and virus is released by several cycles of freeze/thawing. The mixture of Ad.AAV1 and ΔAd.AAV1 particles in the cell lysate is then separated by ultracentrifugation in a CsCl step gradient. Due to its lighter buoyant density, the band containing the ΔAd.AAV1 particles is clearly separated (0.8cm distance) from the band containing full-length virus (Lieber, A., et al. 1999. *J*

Virol 73:9314-24). ΔAd.AAV1 is purified further by an additional CsCl equilibrium gradient and is stored in 10mM Tris pH7.5, 10% glycerol, 1mM $MgCl_2$ in 80°C. In total, the production of 2×10^{13} (genome) particles of ΔAd.AAV1 requires less than 3 hours of work. All functions for ΔAd.AAV1 replication and particle formation are provided from Ad.AAV1 genomes amplified in the same cell. The efficiency of vector production measured on a genome-per-cell-basis is comparable or higher than labor-intensive, newer techniques for rAAV production, which have not yet been proven to be reliable. The estimated ratio of transducing/genome titer for ΔAd.AAV1 is 1:200 (based on SEAP expression at day 3 post-infection), whereas for the average rAAV preparation, it is in the range of $1:10^3$ to $1:10^4$. 1×10^5 confluent SKHep1 cells are infected with different MOIs of rAAV1 (stock: 1×10^{10} genomes per ml), ΔAd.AAV1 (stock: 5×10^{12} genomes per ml), Ad.ADAV1 (stock: 1×10^{13} genomes per ml), and AdAAV1.2ITR (stock: 9×10^{12} genomes per ml), in a volume of 100ml 24 hours after infection, cells are washed, trypsinized, and plated at different dilutions. G418 is added 24 hours after plating and selection is performed for 4 weeks. G418 resistant colonies contain on average $>5\times10^4$ cells (at least 16 cell divisions). A significant number of small colonies visible at 2 weeks post-infection do not survive continued selection, probably due to episomal vector expression. Cells infected with first-generation adenoviruses with MOIs greater than 1×10^4 develop CPE during the first week of selection. The rAAV titer is not high enough to perform infection studies with MOIs greater than 10^4. The colony formation is expressed as percentage of the number of colonies after selection to the number of cells initially seeded for selection (Table I).

TABLE I

Formation of G418 resistant colonies after infection with hybrid viruses in comparison with rAAV.

MOI (genomes per cell)	Formation of G418 resistant colonies in % (SEM) (after 4 weeks of selection)

	rAAV1	.Ad.AAV1	Ad.AAV1	Ad.AAV1 .2ITRs
10^1	0	0	0	0
10^2	0	0	0	0
10^3	2.7 (1.6)	1.3 (1)	5.4 (3.0)	0
10^4	90.8 (7.0)	48.0 (8.9)	12.9 (7.2)	0
10^5	N/A	93.1 (5.4)	3.8 (2.1)	0
10^6	N/A	100	0	0
10^7	N/A	100	0	0

N = 3 (SEM is indicated in parentheses.)

K562 cells are infected with different MOIs of ΔAd.AAVBG (1-10^8 genomes per cell):

Three days after infection, the total number of viable cells (based on Trypan blue staining) and the percentage of infected cells (based on X-Gal staining) are determined for all MOIs. The results are presented in Figure 4A.

Initial integration studies:

K562 cells are incubated with ΔAd.AAVSNori at an MOI of $2x10^5$ genomes per cell and the colonies that formed after 4 weeks of G418 selection are counted in 96 well plates. G418 resistant colonies contain on average $>5x10^4$ cells which means that the original cell underwent at least 16 cell divisions.

Infection studies with .Ad.AAVBG (1-10^8 genomes per cell) on CD34+cells:

Cell infection on CD34+are as described for K562 cells. CD34+ cells are cultured in IMDM supplemented with 20% FCS, kit ligand (stem cell factor-SCF) (100ng/ml), and IL-3 (100ng/ml). Since a number of reports suggest that specific cytokines like GM-CSF or M-CSF which induce stem cell differentiation can stimulate integrin expression and may therefore affect internalization of Ad5 vectors, infection rates are compared with

Ad5 based hybrid vectors on CD34+ cells cultured with and without pre-stimulation with GM-CSF (50ng/ml) or M-CSF (50U/ml). The number of infected cells is counted based on X-Gal staining at day 3 after infection. To test for dose-dependent toxicity, viable cells are counted (based on trypan blue exclusion) at day 3 post-infection. Furthermore, whether high viral doses affect the ability of CD34+ cells to differentiate in methyl cellulose colony assays in presence of IL-3 and SCF is analyzed. The results are expressed as viable cells/X-Gal positive cells vs MOI (see Figure 4B).

DISCUSSION

The above data demonstrate that ΔAd.AAV transduces stably an immortalized human cell line with a low frequency comparable to rAAV, however, transduction rates could be scaled up to 100% by using greater MOIs of ΔAd.AAV1, which is produced at higher titers than rAAV1. In contrast to infection with the first-generation vector, Ad.AAV1, infection with ΔAd.AAV1 is not associated with dose-dependent cytotoxicity because no viral proteins are expressed from these vectors in transduced cells. Furthermore, viral proteins present in the incoming ΔAd.AAV1 particles are not problematic in the dose range use. The comparison of transduction rates of ΔAd.AAV/Ad.AAV1 with the vector lacking AAV ITRs, Ad.AAV-Δ2ITRs, supports the hypothesis that the presence of two intact AAV ITRs is crucial for hybrid vector integration.

The data demonstrate that the leukemia cell line can be infected at ~90% efficiency with Ad5 based hybrid vectors at MOIs $2x10^5$ genomes per cell without significant toxic side effects. However, this dose is still ~100 times greater than the dose necessary to infect 100% of HeLa cell, hepastoma cells, primary hepatocytes and other cell lines generally considered as permissive for Ad5 vector infection.

Since viral DNA in cells infected with $2x10^4$ genomes (or 100 transducing particles per cell) should be lost after 7 cell divisions, the presence of G418 resistant cells in the observed colonies suggests that ΔAd.AAVSNori genomes are integrated into or stably associated with the host genome. Based on the number of G418 resistant colonies one

out of 25,000 ΔAd.AAVSNori genomes integrates stably into K562 cells. This is in agreement with the results obtained earlier with ΔAd.AAV1 in SK Hep1 cells.

The maximal dose used for infection of CD34+ cells ($1x10^8$) results in X-Gal staining of only ~10% of cells independently of GM-CSF/M-CSF. This demonstrates the obvious inability of Ad5 to infect CD34+ cells and is probably caused by the absence of specific receptors and/or integrins on the cell surface. CD34+ cells tolerate a large range of viral doses ($1\text{-}10^7$) without obvious effects on cell viability and total cell number. This is not surprising because in order to develop toxic side effects adenovirus has to enter the cell and express viral genes. Hybrid vectors can be produced at titers of $5x10^{12}$ genomes per ml. Thus, the maximal MOI that can be used for infection (of 10^4 cells) is ~$5x10^8$ (in 100μl storage buffer). Based on the infection studies with ΔAd.AAVBG this dose may not be sufficient to efficiently transduce CD34+ cells and to obtain an appreciable number of G418 resistant colonies.

<u>C. *In Vivo* Properties Of ΔAd.AAV1:</u>

Viral DNA is labeled with BrdU during virus amplification to investigate cellular/nuclear vector uptake in situ. For transduction studies, confluent SKHep1 cells (a human endothelial cell line) are infected with 2000 genomes ΔAd.AAV1 or Ad.AAV1 per cell. BrdU tagged viral DNA is detected in 100% of nuclei at 3 hours post-infection for both viruses indicating efficient cellular and nuclear uptake of hybrid virus DNA.

RESULTS

The ΔAd.AAV1 vector transduces a cell in vitro forming G418 resistant colonies with an efficiency of 17 or 58%, after infection with an MOI of $1x10^3$ or $1x10^4$ genomes per cell, respectively. Approximately $2x10^4$ ΔAd.AAV1 genomes are required to yield one stable transfectant. Since all stable colonies contain integrated ΔAd.AAV1 vector DNA, this number reflects the minimal integration frequency of ΔAd.AAV1 in SKHep1 cells which is comparable with that from rAAV (Rutledge, E. A. et al., 1997, *Journal of Virology,*

71:8429-36). The number of G418 resistant colonies does not necessarily represent the total frequency of integration events because not all integrated copies express neomycin phosphotransferase, due to chromosomal position effects or incomplete integration.

The absence of adenoviral gene products in ΔAd.AAV1 transduced cells at day 3 post-infection is demonstrated by immunofluorescence with antibodies to the major late proteins (hexon, fiber) and early proteins (DBP.E4-orf6). Expressed adenoviral proteins are detected only in cells infected with Ad.AAV1. The fact that cells infected with ΔAd.AAV1 do not express potentially cytotoxic adenoviral proteins is important.

While an MOI of $1x10^4$ genomes per cell of the first generation vector Ad.AAV1 induce cytopathic effects in SKHep1 cells at day 3 p.i., no toxic side effects are observed when SKHep1 cells are infected with ΔAd.AAV1 at a dose of up to $1x10^8$ genomes per cell. Since the transduction efficiency is clearly dose dependent, ΔAd.AAV1 (which can be produced at titers of $>5x10^{12}$ genomes/ml) is able to stably transduce Ad5 permissive cell lines or tissues with a 100% efficiency without associated toxicity.

Southern analysis indicates that ΔAd.AAV1 integrates randomly as head-to-tail tandem repeats into the host cell genome via the right AAV ITR, whereas the other junction with the chromosomal DNA is variable and occurs somewhere within the transgene cassette. In order to confirm the integrated status of ΔAd.AAV1 DNA, high-molecular-weight chromosomal DNA is separated by pulse field gel electrophoresis (PFGE), followed by Southern analysis with a SEAP specific probe (Figure 3). Undigested DNA from control SKHep1 cells give an endogenous SEAP signal that co-migrates with chromosomal DNA just below the well (lanes 1 and 5). No high-molecular weight episomal forms of ΔAd.AAV1 DNA are detected, whereas a distinct 35 kb band is visible in DNA from SKHep1 cells isolated 3 days after infection with first generation adenovirus, Ad.AAV1 (lanes 4 and 13). Digestion with EcoRI reveals the 4.4kb fragment, which is specific for integrated tandem copies of the AAV cassette (lanes 8 and 12). To eliminate the possibility that chromosomal DNA is trapped in the well, DNA samples are digested with intron-encoded endonucleases PI-SceI or I-CeuI (Gibco-BRL, Grand Island, NY) with a

sequence specificity or more than 11bp or 9bp respectively. Digestion with PI-SceI yields a >2mb endogenous SEAP signal in SKHep1 cells (lane 2) and an additional signal in the range of ~1mb in G418 resistant colonies transduced with ΔAd.AAV1 (lane 7). I-CeuI digestion results in a smear between 250-1000 kb in ΔAd.AAV1 transduced SKHep1-cells (lanes 10, 11) indicating random integration, whereas a high-molecular weight band specific for the endogenous SEAP gene is observed in control SKHep1 cells (lane 9).

One day after intraportal infusion of 1×10^{12} ΔAd.AAV1 genomes in C57Bl/6 mice, BrdU labeled vector genomes can be detected in 85% hepatocytes (Lieber, A., et al. 1999. *J Virol* 73:9314-24). Hepatocellular DNA analysis performed at 2 months post-infusion reveals ΔAd.AAV1 DNA integrated with an average of 0.5 copies per cell into the mouse genome (Lieber, A., et al. 1999. *J Virol* 73:9314-24). To assess potential side effects of intraportal ΔAd.AAV1 infusion, serum glutamic pyruvic transaminase (SGPT), a sensitive marker for hepatocellular injury, is measured for 7 consecutive days post-infusion in combination with histological analysis of liver sections. No significant elevation in SGPT levels, or histological abnormalities are detected after intraportal infusion of 1×10^{12} or 1×10^{13} ΔAd.AAV1 genomes, whereas infusion of the same dose of full-length Ad.AAV1 vector is associated with severe hepatoxicity or fatal outcome. This suggests that the dose of ΔAd.AAV1 administered to mice can be increased to obtain higher transduction efficiencies in vivo without adverse side effects, which is not possible for first generation adenoviruses. Importantly, ΔAd.AAV1 transduced quiescent hepatocytes in vivo, which suggests that integration of hybrid vector DNA may not require cell proliferation. Recently, more detailed in vivo transduction studies with Ad.AAV1 and ΔAd.AAV1 have been performed in Balb/c mice to study whether the absence of adenoviral gene expression in cells infected with ΔAd.AAV1 can avoid an anti-viral immune response and can prolong vector persistence. In this mouse strain, vector DNA is cleared from the liver at 4-6 weeks after infusion with first generation adenoviruses, mostly due to a CTL response against viral proteins produced in transduced cells. Vector DNA is analyzed by genomic Southern Blot of hepatic DNA at 12 weeks

after infusion of $1x10^{12}$ genomes Ad.AAV1 or ΔAd.AAV1. At this time point, no vector specific signal is detectable in hepatic DNA from mice infused with the first generation vector Ad.AAV1, while ~0.3 copies of ΔAd.AAV1 genomes per cell are present in livers of mice that received the hybrid vector, again indicating the superior in vivo properties of the hybrid vector.

D. Effects of Rep Coexpression on ΔAd.AAV Integration

Rep expression after plasmid transfection:

In order to test whether Rep expression enhances site-specific integration of ΔAd.AAV1 in human cells, a series of Rep expression plasmids are constructed.

METHODS

The Rep ORF 68/78 (nt 285-2313) including the internal p19 and p40 promoters is obtained from pAAV/Ad (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia) by digestion with BsaI/BsrI. This fragment deleted for the AAV p5 promoter is cloned via adapter linkers under RSV or PGK promoter in front of the bovine growth hormone polyadenylation signal (bPA) into pAd.RSV or pAd.PGK (Lieber, A., and Kay, M.A., 1996, *J. of Virology*, 70, 3153-3158; Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) correspondingly.

RESULTS

The resulting plasmids (pRSVrep, pPGKrep) are transfected into 293 cells or SKHep1 cells, most of the Rep proteins expressed from the heterologous promoters (RSV or PGK) are Rep 68 and Rep 78, while transfection of the rep gene under aP5 promoter (pAAV/Ad) results in predominant Rep 52/40 expression. Thus, transfection of pRSVrep and pPGKrep is more pronounced suggesting a strong transactivation of AAV promoters

by Ela which is produced in 293 cells. This result indicates that minimum expression of rep proteins is necessary to avoid interference with adenovirus replication.

Rep-mediated site-specific integration of ΔAd.AAV1.

The potential for site-specific integration is an important characteristic of the novel Ad.AAV vectors of the invention. In an embodiment of the invention, integration of the ΔAd.AAV is directed by co-infection with Ad AAV expressing the rep 78 protein to achieve site-specific integration in the AAVS1 site on human chromosome 19. For this type of site-specific integration to occur in cells other than 293 cells, E4 ORF6 expression is required. The co-infection of ΔAd.AAV, ΔAd. rep 78, and ΔAd. E4-orf6 allows for site specific integration of the ΔAd.AAV transgene cassette. The ΔAd. rep78 and the ΔAd. E4-orf6 genomes are degraded soon after transduction, thus avoiding potential side effects. Site-specific integration is preferred over random integration, which is seen with rAAV and ΔAd.AAV, in order to reduce the risk of insertional mutagenesis.

A preliminary test can be performed to confirm the functional activity of Rep 68/78 expressed from pRSVrep to mediate site-specific integration of ΔAd.AAV1 (Figure 5 and 6). Human SKHep1 cells are transfected with pRSVrep or control plasmid (pRSVbGal (Lieber, A., et al., 1995, *Human Gene Therapy*, 6, 5-11) (transfection efficiency was ~20%), followed by infection with ΔAd.AAV (2000 genomes per cell). Three days after infection, cells are trypsinized, embedded in agarose, lysed in situ, digested with I-CeuI (an intron-encoded endonuclease with a recognition sequence of more than 10nt), subjected to pulse file gel electrophoresis in 1% agarose gel, and analyzed by Southern Blot. Hybridization with a probe covering the AAVS1 integration site (1.7kb EcoRI/BamHI fragment from the chromosome 19 locus (Samulski, R. J. et al., 1991, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia)) reveals an AAVS1-specific band (~240kb) in I-CeuI digested DNA from cells after control plasmid transfection (pCo) + ΔAd.AAV1 infection. An additional signal in the range of 280kb appears in rep expressing cells infected with ΔAd.AAV1

(pRSVrep + VAd.AAV1) indicating a site-specific insertion into the AAVS1 site in a certain percentage of cells. The presence of vector DNA in this 280kb band is confirmed by rehybridization of the same filter with a transgene (SEAP) specific probe. Randomly integrated ΔAd.AAV1 vector appears as a diffuse SEAP signal in the range 280-680kb (pCo+ΔAd.AAV1, pRSVrep+ΔAd.AAV1). The specific ~1.9 mb band on blots hybridized with the SEAP probe represents an I-CeuI fragment containing the endogenous human SEAP gene.

Incorporation Rep 68/78 function into hybrid vectors to stimulate site-specific integration

Rep overexpression inhibits adenovirus DNA replication, prohibiting the generation of rep expressing Ad vectors using conventional strategies. To solve this problem, significant Rep 68/78 expression from the hybrid vector in virus producer (293) cells must be prevented while maintaining transient Rep expression in target cells (HSC) to mediate site-specific integration. Our hypothesis is that the specific structure of the ΔAd.AAV hybrid virus can be used to bring the rep gene 68/78 into a transcriptionally active position under control of a HSC specific promoter only at late stages of virus replication in 293 cells. This will allow amplification of the hybrid vector in 293 cells, generating high titer virus which activates the incorporated Rep 68/78 functions only in HSC. The general outline of our strategy to produce Rep expressing hybrid vectors is illustrated in Figure 7. The rep/transgene cassette is assembled based on the left-hand shuttle plasmid used for recombinant adenovirus production. The gene encoding Rep 68/78 is cloned in 3'□5' orientation in front of a transgene expression cassette flanked by AAV ITRs. Between the transgene cassette and the right AAV ITR an HSC-specific promoter is inserted with direction towards the adenoviral E2, E3, and E4 genes. The recombinant genome is produced by recombination in E.coli and transfection into 293 cells generates virus (Ad.AAV-rep). The specific structure of ΔAd.AAV with duplicated sequences flanking the AV ITRs is used to bring the rep gene into a transcriptionally active position under control of a HSC specific promoter only during late stages of viral DNA replication in 293 cells. During amplification of Ad.AAV-rep, the smaller genome ΔAd.AAV-rep is formed and packaged into particles, which can be separated by ultracentrifugation in CsCl gradients. The specific structure of ΔAd.AAV-rep brings the

rep gene into 5'↔3' orientation in relation to the HSC specific promoter, allowing rep transcription in target cells. After transduction of HSC with purified ΔAd.AAV-rep particles, rep expression is activated and mediates rescue of the AAV-ITR/transgene cassette from the adenoviral vector backbone and site-specific integration. The hypothesis is that Rep-mediated integration into AAVS1 occurs via the right or both AAV/ITRs causing the rep gene to become separated from the hepatocyte-specific promoter once the vector is integrated (Figure 7). Therefore, rep expression should be only transient without critical cytotoxic side effects on the host cell.

Promoters that can regulate rep expression:

Potential candidate promoters to drive rep expression with high specificity for HSC and minimal activity in 293 cells are the 454nt CD34 promoter (Krause, D.S., et al., 1997, *Experimental Hemotology*, 25, 1051-1061; Yamaguchia, Y. et al., 1997, *Biochimica et Biophysica Acta.*, 1350:141-6), the 300nt HS 40 enhancer (Chen, H.L., et al., 1997, *Nucleic Acids Res.* 25, 2917-2922) or a 3kb CD34 enhancer (May, G. et al., 1995, *EMBO J.*, 14:564-74) in combination with an initiator, or the HIV LTR. An optimal promoter is selected based on studies of transient reporter gene expression after plasmid transfection in 293 cells and hepatocytes. All promoters to be tested are cloned in front of the human α_1-antitrypsin (hAAT)- bovine growth hormone polyadenylation signal (bPA) into the adenoviral shuttle plasmid pCD2 (pAd.-hAAT). Promoter activity can be tested in transient plasmid transfection assays in CD34+ and 293 cells. The promoter with the highest hAAT levels in CD34+ or K562 cells and the lowest hAAT expression in 293 cells is selected for further studies. If high background expression in 293 cells from these promoters is seen, insulators to shield HSC-specific promoters from the Ela enhancer which is still present in Ad shuttle plasmids can be utilized.

Rep genes:

The large Rep 68/78 proteins are sufficient to mediate rescue and site-specific integration. Unregulated Rep 52 and Rep 40 expression from the AAV p19 promoter

located within the ORF of Rep 68 and 78 must be prevented because production of these smaller Rep proteins in 293 cells will affect cell viability and adenoviral DNA synthesis. To do this, constructs obtained from Surosky et al., containing a mutated Rep 52/40 start codon to express Rep 68 and 78 individually under CMV promoter can be used. The 293 cells transiently expressing Rep68 or Rep 78 from these constructs can be coinfected with ΔAd.AAV1 (infection 24 hours after pCMVRep transfection, MOI 2×10^5 genomes/cell). Three days after ΔAd.AAV1 infection, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE as described earlier. Efficient Rep mediated excision of the AAV cassette and site-specific integration without flanking adenoviral sequences are expected and the plasmids pCMVRep68 or pCMVRep78 can be used as a source for the corresponding rep genes and clone them into hybrid vectors.

Vectors:

The rep/transgene cassette can be assembled based on pXCJL (Microbix, Toronto). A set of control hybrid vectors can be generated with the AAV-ITR-transgene cassette only without the rep gene. The recombinant Ad.AAV-rep genome can be generated by recombination of the left hand shuttle plasmids with pCD1, a pBHG10 (Microbix, Toronto) derivative, which contains the Ad5 genome deleted for the E1/E3 regions in $recA^+$ E. coli (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Compared to the standard technique based on plasmid recombination in 293 cells, this approach has the advantage that plaques with recombinant virus appear 3 times faster and the production of illegitimate recombinants is minimized. This allows efficient viral DNA amplification and packaging to occur before Rep expression reaches levels that are potentially inhibitory for adenoviral replication. The critical variables in maximizing the output of the vector deleted for all adenoviral viral genes are the initial multiplicity of infection and the time of harvesting. These parameters can be optimized for production of ΔAd.AAV-rep hybrid vectors. A number of ΔAd.AAV vectors can be constructed incorporating rep gene. Cryptic promoter and enhancer elements present in the 5'-342nt of the adenoviral genome can interfere with transgene expression from the heterologous promoters. This is crucial for the strategy to avoid rep expression from ΔAd.AAV-rep genomes in 293 cells.

To ensure efficient transgene expression, insulator fragments such as the chicken beta-globin insulator can be used with a selected promoter, constitutive or inducible.

Rep protein co-packaging:

As an alternative to producing hybrid vectors containing the rep 68/78 gene, studies are designed to see whether Rep protein can be co-packaged into ΔAd.AAV capsids and whether these co-packaged Rep molecules are sufficient to mediate rescue and site-specific integration of the AAV-ITR-transgene cassette. Our hypothesis is that the Rep 68/78 binds to the Rep binding site (RBS) present in double-stranded ΔAd.AAV genome and that this complex is co-packaged into adenoviral capsids which are spacious enough to accommodate extra proteins. Based on protein/DNA ratio analysis performed previously in purified particles that only one 5.5kb ΔAd.AAV1 genome is packaged per capsid. This is confirmed by electron-microscopy of ΔAd.AAV1 particles, which reveals only spotted electron-dense staining associated with viral cores and extended free luminal space (see Figure 2).

293 cells are transfected with plasmids expressing Rep 68/78 under the CMV promoter and the kinetics of rep expression is determined by Western Blot with cell lysates collected at different time points after transfection. Next, these 293 cells are infected with Ad.AAV (MOI 1, 10, 100 pfu/cell) at specific time points after transfection of Rep plasmids depending on the Rep expression kinetics (e.g. 3, 6, 12, 24 . . . hours after transfection). It is important to time Ad.AAV infection exactly because viral DNA replication must be taking place or finished before Rep production reaches peak levels. In general, adenovirus DNA replication in 293 cells (infected with MOI 10) is maximal at 18 hours post-infection, followed by production of structural proteins, packaging of viral genomes, and breakdown of cellular membrane structures (which is concluded ~36-48h p.i.) (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia; van der Vliet, B., 1995, *In* w. Doerfler, et al. (eds.) vol. 2 p. 1-31, Springer-Verlag, Berlin). Viruses are collected 48h after infection and banded by CsCl ultracentrifugation. Viral material from purified bands corresponding to

ΔAd.AAV is lysed, DNAse-treated (to liberate DNA associate Rep) and subjected to immunoprecipitation-Western Blot with Rep specific antibodies to detect co-packaged Rep. Based on theoretical calculations assuming that two Rep molecules bind per Ad genome, ~1-10ng Rep proteins is expected from Lysates of 10^{10} particles, which is within the range of detectability by Western Blot. Alternatively, co-packaged Rep may be detected based on its functional activity to mediate rescue and site-specific integration of the AAVITR transgene cassette. To test whether functional Rep protein is co-packaged into hybrid vector particles, CsCl purified ΔAd.AAV1 particles generated in 293 cells co-expressing Rep after Ad/AAV1 infection (ΔAd.AAV1+Rep) can be used for transduction studies. Three days after ΔAd.AAV1+Rep infection of the human cell line K562, cellular DNA is analyzed for AAVS1-specific integration events by PCR and PFGE. If efficient Rep-mediated site-specific integration of excised AAV cassettes is successful, then other ΔAd.AAV+Rep hybrid vectors with β-Gal and SNori as transgenes can be produced.

Integration studies with Rep vectors in erythroid cells:

The hypotheses behind the rational of a rep-expressing hybrid vector (ΔAd.AAV-rep) are: (1) transient Rep co-expression from ΔAd.AAV-rep vectors can enhance site-specific vector integration in human cells and (2) integration occurs via the AAV ITR(s) without the rep gene, which is placed outside the AAV cassette, thus eliminating rep expression upon vector integration. To test hypothesis 1, transduction frequencies of ΔAd.AAV/rep versus ΔAd.AAV vectors can be compared based on the formation of G418 resistant colonies and quantify site-specific integration events at different time points after infection of human and mouse cells by PFGE and PCR. To test hypothesis 2, the structure of integrated vector in transduced cell populations and single clones can be delineated by Southern analysis and by sequencing of vector/chromosomal DNA junctions. These studies can be performed with ΔAd.AAV-rep,ΔAd.AAV, and ΔAd.AAV+Rep (copackaged protein) in human K562 or HEL (for AAVS1 integration) and mouse MEL cell lines.

Cells infected with ΔAd.AAV-SNori, ΔAd.AAV-SNori+Rep or ΔAd.AAV-Snori-rep can be subjected to G418 selection. The number of G418 resistant colonies determined after 4 weeks of selection in relation to the number of initially infected cells. The selection process for colonies that did not survive continued selection due to potential rep-mediated cytotoxcicity or episomal vector expression can be monitored. If rep expression from ΔAd.AAV-SNori rep does not affect cell viability and proliferation, then more G418 resistant colonies should appear in ΔAd.AAV-SNori-rep and ΔAd.AAV-Snori+Rep. The structure of integrated vector can be determined by Southern Blot and sequencing of integration junctions.

To uncover a potential selection bias against rep producing cells after transduction with ΔAd.AAV/rep, site-specific and random vector integration events can be quantitated in cellular DNA isolated from cell populations at different time points after infection (e.g. 0.5, 1, 3, 7, 14 days). To do this, the techniques based on PFGE-Southern can be utilized. It is expected that the signal(s) for AAVS1-specific integration in ΔAd.AAV/rep infected human cells increases during the first days after infection and then remains constant over time.

In a separate study, the integration status of vector DNA (analyzed by PFGE or PCR) and the number of integrated copies (analyzed by Southern Blot) with the expression level of β-galactosidase in single clones transduced with β-Gal hybrid vectors (ΔAd.AAV-BG, ΔAd.AAV-BG+Rep, or ΔAd.AAV-BG-rep) can be correlated. Together with data obtained in the studies described in the Specification, this allows assessment of whether transcriptional silencing is associated with site-specific vector integration into the AAVS1 site.

It is not clear a priori whether the specific Rep function for vector rescue, concatemerization, and integration can efficiently occur in non-S-phase or non-dividing cells. To test whether ΔAd.AAV^{fx} , ΔAd.AAV or ΔAd.AAV-rep/+Rep vectors can integrate into non-dividing cells, transduction studies in cell cycle arrested cell cultures can be performed as described earlier.

DISCUSSION

The establishment of stable cell lines expressing Rep 68/78 at detectable levels is not possible, which is probably due to rep mediated cytotoxicity. Therefore, it is not possible to perform long-term transduction studies (e.g. G418 selection or studies in single clones) in combination with ectopic rep expression. Moreover, due to the inhibitory effect of rep on adenovirus replication, it is currently not possible to generate adenoviral vectors expressing rep under the RSV or PGK promoter.

Taken together, this indicates that co-expressed Rep may stimulate site-specific transgene integration.

E. A Detailed Study Of Transduction/Integration Of Hybrid Vectors In Erythroid Cell Lines:

In order to improve transduction and integration frequencies of the hybrid vectors into erythroid cell lines, a detailed study comparing various hybrid vectors have to be carried out as described below. The transduction studies are performed in K562 cells which is considered to be an adequate model to study gene transfer vehicles into erythroid cells (Floch, V., et al., 1997, *Blood Cells, Mol. and Diseases.*23, 69-87). The optimal vectors should be able to integrate into the cellular genome with a high frequency, determined by Pulse field gel electrophoresis (PFGE) and Southern blot as described in Example 4. In addition, the results from the following studies will serve to evaluate whether a given hybrid vector needs to be modified for site-specific integration in the host genome.

Sequencing of integration junctions:

The ultimate proof for vector integration is the sequencing of junctions between SNori vector DNA and chromosomal DNA. Furthermore, this clarifies the question whether the AAV ITRs represent the substrate for integration. Specifically, DNA from clones with

known ΔAd.AAVSNori integration structure (analyzed by Southern Blot) digested with EcoRI, which does not cut within the SNori cassette. The resulting fragments are circularized and transformed into a specific E. coli strain (according to the protocol described by Rutledge and Russell (Rutledge, E. A. et al., 1997, *Journal of Virology*, 71:8429-36)). Kanamycin resistant bacterial clones should contain the integrated SNori cassette. Flanking chromosomal DNA in rescued plasmids can be sequenced with primers specific to the transgene.

To confirm vector integration in a small number of transduced cells, genomic DNA is extracted and digested with EcoRI. EcoRI fragments are ligated to linkers containing a specific primer binding site and are then digested with NotI, religated and propagated in *E. coli*. Plasmid DNA from a representative number of bacterial clones is sequenced to determine the vector/chromosomal DNA junctions.

Dose dependent toxicity:

In order to test that the transduction frequence is dose-dependent and ΔAd.AAV vectors, which are devoid of all adenoviral genes, could be used to infect cells at higher doses with less cytotoxicity than first generation adenovirus, K562 cells are infected with different MOIs ($1\text{-}10^8$) of ΔAd.AAVBG and the first generation vector Ad.AAVBG (which contains the same β-Gal expression cassette). At day 4 post-infection, the total number of cells, the percentage of viable cells (based on trypan blue exclusion) and the percentage of X-Gal positive cells are counted. A fraction of infected cells are quantified for β-Gal expression using the Galacto-Light kit. The level of transgene expression is expected to be comparable between the two vectors. K562 cells are predicted to tolerate higher doses of ΔAd.AAVBG better than Ad.AAVBG which express viral genes.

Integration frequency with and without G418 selection:

In order to investigate the integration frequency of the different vectors and to confirm that AAV ITRs present in double-stranded adenoviral DNA genomes can mediate vector

integration with a frequency comparable to rAAV vectors. integration studies are performed based on the formation of G418 resistant colonies with ΔAd.AAVSNori, AdSNori, Ad.SNoriITR, and rAAVSNori after infection with $2x10^5$ and $2x10^6$ genomes per cell (Fig. 8). After infection, cells are plated in 96 well plates under limiting dilution and selected with G418 to estimate the frequency of formation of G418 resistant colonies. Another set of cells is plated without G418. A representative number of clones (w/ and w/o G418 selection) are expanded to $>10^6$ cells (after 3-4 weeks of culture) and analyzed for the presence of viral DNA by Southern Blot as well as PFGE analysis to discriminate between episomal vector DNA and vector genomes stably associated with chromosomal DNA. This allows us to estimate the integration frequency of the different vectors, to assess the effect of G418 selection on integration, and to consider position effects on neo expression in calculating the total integration frequency. Integrated vector copies with a frequency of at least $1x10^{-4}$ is predicted only for ΔAd.AAVSNori and rAAVSNori. The total number of colonies may be lower in both the first generation vectors, Ad.AAVSNoriITR and Ad.SNori, due to the toxic effects of expressed adenoviral proteins; however, a higher integration frequency is predicted for the vector containing the AAV ITRs (Ad.AAVSNoriITR).

Kinetics of integration:

Compared to rAAV, the double-stranded nature of entering ΔAd.AAV genomes provides more protection against degradation. Furthermore, the synthesis of transcriptionally active double-stranded intermediates from single-stranded genomes, which is considered a limiting step in rAAV transduction, is not required in ΔAd.AAV transduction. Thus, the lag phase between infection and expression seen with rAAV vectors, which is causally linked to double-strand synthesis/integration may be shorter or absent in infections with ΔAd.AAV vectors. Furthermore, it was demonstrated earlier that a 9kb mini-adenoviral genome packaged into adenoviral particles is only short lived and completely degraded by day 3 post-infusion. In contrast, transduction with the 5.5kb ΔAd.AAV1 (Figure 1) genome allows for long-term expression, suggesting that either

AAV ITRs can stabilize the viral genome as an episome until it is integrated or integration occurs shortly after infection.

The status of vector DNA can be examined in K562 cells at different time points after infection with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, or rAAVSNori (MOI $2x10^5$). Infected cells are harvested at 1 hour, 5 hours, 1 day, 3, 7, and 14 days after infection and chromosomal DNA is analyzed by PFGE followed by hybridization with a transgene specific probe. This technique allows us to distinguish between episomal vector DNA, which appears as a distinct 5.0kb band and integrated DNA. Furthermore, extra chromosomal high-molecular weight vector concatemers can be detected. In the case of random integration, after digestion of chromosomal DNA with I-CeuI or PI-SceI, vector-specific signals in the range of 1-2mb should be seen. The intensity of episomal and integrated vector signal is quantified for each time point using phosphoimager analysis. This gives information about the kinetics of hybrid vector integration in a population of infected K562 cells and the intracellular stability of hybrid vector genomes.

Structure of integrated vector DNA and integration junctions with chromosomal DNA:

ΔAd.AAV1 integrates as concatemer/s randomly into host DNA as shown previously. How many vector copies are present in one concatemer and whether the extent and the kinetics of tandem-formation are dose dependent still remain unclear. Another unanswered question is how ΔAd.AAV integrates: whether one or both ITRs are involved, whether the integrated ITRs are still intact, and whether adenoviral sequences integrate as well. These issues are important for the strategy to include rep genes into the hybrid vector genome. Moreover, if intact AAV ITRs are present within integrated vector copies, helper virus (adenovirus or HSV) infection in vivomay mobilize the integrated AAV-ITR vector cassette and affect stability of transgene expression.

To answer these questions, K562 cells can be infected with ΔAd.AAVSNori, AdSNori, Ad.AAVSNoriITR, and rAAVSNori at MOIs $2x10^5$, $2x10^6$, or $2x10^7$ genomes per cell. Infected cells are plated in 96 well plates in the presence or the absence of G418. The

latter is included because G418 may cause amplification of integrated vector DNA (Rutledge, E. A. et al., 1997, *Journal of Virology,* 71:8429-36). Genomic DNA from isolated clones can be analyzed by regular Southern Blot as described in the Examples Section to confirm the presence of vector concatemers and calculate the number of integrated vector copies. More informative is the sequencing of integrated vector copies and their junctions with chromosomal DNA. The structure of integration junctions can be delineated using the role of AAV ITRs in vector integration and the extent of insertional mutagenesis after transduction. This data provides information about the potential risks of hybrid vector used in clinical trials.

Transduction of cell cycle arrested cells:

The ultimate target for the hybrid vectors described in the Specification are quiescent hematopoietic stem cells. We hypothesize that the double-stranded nature of ΔAd.AAV genomes and specific nuclear import mechanisms may allow for the transduction of non-dividing cells. This is in part supported by the transduction studies with ΔAd.AAV1 in quiescent hepatocytes in vivo. To confirm this data, primary fibroblasts can be forced to enter the G_0 phase by serum/growth factor starvation before infection with the hybrid vectors according to a protocol described by Russell (Russell, D. et al., 1995, *PNAS,* 92:5719-23). Cells are maintained for three days after infection under serum/growth factor deprivation. At this time point, genomic DNA is isolated and analyzed for integration events by PFGE in comparison with growing cells. Another series of integration studies can be carried out on K562 cells arrested in the G_1/S phase of the cell cycle with aphidicolin (added 1 day before and maintained several days after infection with hybrid vectors - depending on the integration kinetics studies described earlier). To investigate whether DNA damaging agents increase the transduction frequency of hybrid vectors, cell-cycle-arrested K562 cells or primary fibroblasts can be treated with cisplatinum or ^{3}H-thymidine prior to virus infection according to a protocol described by Alexander and Russell (Alexander, I. E. et al., 1994, *J. Virol.,* 68:8282-87; Russell, D. et al., 1995, *PNAS,* 92:5719-23). Furthermore, the effect of chromosomal DNA decondensation on the transduction efficiency of hybrid vectors can be studied in arrested

cells after treatment with puromycin, staurosporin, Hoechst 3328, distramycin, or vandate.

F. Improvements in ΔAd.AAV Production and Purification

To inhibit packaging of full-length genomes a modified form of I-Sce I, a yeast mitochondrial intron-endonuclease with a non-palindromic 18-bp recognition sequence is expressed in 293 cells. Constitutive expression of this enzyme in mammalian cells is not toxic, possibly due to either the lack of I-SceI sites in the genome or sufficient repair of them (Rouet P. et al, 1994, *PNAS*, 91:6064-8). The yeast I-Sce I is modified with an SV40 T-antigen nuclear localization signal and an optimal Kozak sequence to enhance its functionality in mammalian cells (Rouet P. et al, 1994, *PNAS*, 91:6064-8). For another yeast endonuclease it was shown that a recognition site within an transduced Ad genome was efficiently (30% of all transduced genomes) when expressed in human A549 cells. Importantly, the expression of E4 ORF6 and ORF3 expressed from the transduced Ad genome inhibited double-strand break repair mediated by the endonuclease (Nicolas, A. L. et al, 2000, *Virology*, 266:211-24). This is consistent with the observations by others where these E4 proteins prevent concatemerization of the viral genome (Boyer, J. et al, 1999, *Virology*, 263:307-12). Based on this, packaging of full-length virus containing a I-SceI recognition site is reduced in 293 cells constitutively expressing I-Sce I. The 18mer I-Sce site is inserted into the E3 region of the Ad.IR vectors. These vectors are generated and amplified in 293 cells followed by a large-scale infection of 293 cells expressing I-SceI. Alternatively, an expression cassette for the endonuclease XhoI is inserted into the E3 region of Ad.IR or Ad.AAV vectors. The XhoI gene will be modified for optimal function in mammalian cells. Vectors expressing XhoI are generated and amplified in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Nelson, J. E. et al, 1997, *J. Virol.*, 71:8902-7), which mediates the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG. This protects the viral and cellular genome from XhoI cleavage. Methylated Ad vectors are produced at high titers. ΔAd.AAV vectors are then obtained by large-scale infection of 293 cells with the Ad.AAV-XhoI vectors. At this stage the viral

genome is not methylated and is digested at the XhoI sites. XhoI sites present within the transgene cassette are deleted by site-directed mutagenesis without altering the amino acids sequence. (XhoI is accumulated only at late stages in virus replication and should act only upon a large part of Ad DNA when replication is completed. In addition, ultracentrifugation optimizes the separation between ΔAd.IR and ΔAd.IR particles (Blague, C. et al., 2000, *Blood*, 95:820-8).

EXAMPLE II

MODIFIED FIBER PROTEIN

A. Test the Infectivity of Different Human or Animal Serotype on Human Bone Marrow Cells.

Since the amino acid sequence of the fiber knob region varies considerably among the ~50 known serotypes, it is thought that different adenovirus serotypes bind to different cellular receptor proteins or use different entry mechanisms (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology*, vol. 2 Lippincott-Raven Publisher, Philadelphia; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14; Defer, M., et al., 1993, *J. of Virology*, 64, 3661-3673). Although most adenoviruses contain RGD motifs in the penton base proteins, there are a number of serotypes (e.g. Ad 40, 41) without this conserved sequence. These types may use integrin □v-independent pathways for virus internalization (Davison, A.J., et al., 1993, *J. Mol. Biol.*, 234, 1308-1316; Mathias, P. et al., 1994, *Journal of Virology*, 68:6811-14). To test whether other Ad serotypes can infect stem cell subpopulation present in human bone marrow, studies with a series of different human Ad serotypes and animal viruses can be performed (see Table II). As a means to verify efficient transduction with Ad serotypes, viral DNA is tagged before infection and the presence of viral genomes in the nuclei of transduced cells is investigated. Furthermore, whether viral DNA is replicated in transduced cells can be analyzed as indirect proof for early viral gene expression. A direct detection of expressed viral proteins is impossible due to the unavailability of antibodies against all the serotypes included in this study. Simultaneously with the infection assay, transduced

human bone marrow cells can be analyzed for morphological and immunohistochemical features characteristic of HSC or progenitor subpopulations. For retargeting, serotypes which are able to infect CD34+ subsets of bone marrow cells at the lowest MOI are selected. As the next step, the fiber gene is PCR-cloned from serotypes with potential HSC/CD34+ tropism and inserted into standard shuttle plasmids for Ad5 vector generation replacing the Ad5 fiber gene using an E. coli recombination system (Figure 9).

METHODS

Cells and viruses:

HeLa (human cervix carcinoma, ATCC CCL-2.2), CHO (chinese hamster ovary, ATCC CCL-61), K562 (human hematopoietic, ATCC 45506), HEp-2 (human larynx carcinoma, ATCC CCL-23), 293 (human embryonic kidney, Microbix, Toronto Canada) cells were maintained in DMEM, 10% FCS, 2 mM glutamine, and Pen/Strep. Culture media for CHO cells was supplemented with 200μM asparagine and 200μM proline. Human CD34+-enriched bone marrow cells were purified from peripheral blood after mobilization using MiniMACS VS^+ separation columns (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. Aliquots were stored in liquid nitrogen. Sixteen hours before the experiment, cells were recovered from the frozen stock and incubated overnight in IMDM media, supplemented with 20% FCS, 10^{-4} M β-mercaptoethanol, 100 μg/ml DNaseI, 2 mM glutamine, 10 U/ml IL-3, and 50 ng/ml stem cell factor (SCF) or 2 ng/ml thrombopoietin (Tpo). The purity of CD34+ preparations was verified by flow cytometry and was consistently greater than 90%.

Flow cytometry:

Adherent cells (CHO, HeLa) grown in non-tissue culture treated 10 cm dishes (Falcon, Franklin Lakes, NJ) were detached by treatment with 1mM EDTA and washed three times with wash buffer (WB), consisting of PBS supplemented with 1% FCS. Cells

grown in suspension (K562, CD34+) were washed three times with WB. After washing, cells were resuspended in WB at 2 x 10^6 cells/ml. 2 x 10^5 cells were incubated in WB for 1 h at 37°C with monoclonal antibodies specific for α_v-integrins [L230, ATCC: HB-8448, (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795) (1/30 final dilution), CAR [RmcB (Bergelson, J. M., et al. 1997. *Science*. 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology*. 62:1647-1652) (1/400 final dilution)], or BrdU [(Amersham, Arlington Heights, IL) (1/100 final dilution)]. Subsequently, cells were washed with WB, and incubated with fluorescein isothiocyanate (FITC)-labeled horse anti-mouse IgG antibodies [(Vector Labs., Burlingame, CA) (1/100 final dilution)] or phycoerythrin (PE)-labeled goat anti-mouse IgG antibodies [(Calbiochem, La Jolla, CA) 1:100 dilution] for 30 min at 4°C. After incubation with secondary antibodies, cells were washed two times with WB and 10^4 cells per sample were analyzed in duplicate by flow cytometry.

For the analysis of CD34 and c-kit expression on transduced CD34+-cells and for fluorescent activated cell sorting (FACS), purified human CD34+ cells were incubated with phycoerythrin(PE)-conjugated anti-CD34 monoclonal antibodies (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA) or with PE-labeled anti-CD117 (c-kit) monoclonal antibodies (MAb 95C3, Immunotech, Beckman Coulter, Marseille, France) according to the manufacturer's protocol followed by flow cytometry analysis. All analyses and sortings were performed on a FACStar Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with 488nm argon and 633 nm HeNe lasers. For analysis of c-kit expression and FACS purification of CD34+/c-kit+ cells, SCF was not added to the media during culturing of CD34+ cells.

RESULTS

CAR/α_v-integrin expression on test cells:

It is generally accepted that CD34+ cells possess bone marrow repopulating activity. Therefore, we used human CD34+ cells as the target for our studies towards identifying Ad serotypes with HSC tropism and constructing new viral vectors. Studies were

performed on mobilized, CD34-positive, peripheral blood cells from one donor under conditions which are known to retain CD34+ cells in a quiescent stage (Leitner, A., et al. 1996. *Br.J.Haematol.* 92:255-262; Roberts, A. W., Metcalf, D. 1995. *Blood.* 86:1600-1605). More than 90% of purified cells were CD34 positive by flow cytometry. Furthermore, we included into our Ad tropism studies the cell line K562, which is considered to be an adequate model for studying gene transfer into human hematopoietic cells (McGuckin, et al. 1996. *British Journal of Haematology.* 95:457-460). HeLa cells, which are readily infectible by Ad5, and CHO cells, which are refractory to Ad5 infection (Antoniou, M. et al., 1998, *Nucleic Acid Res.,* 26:721-9), were used as positive and negative control cell lines, respectively.

For Ad5, both, binding to the primary receptor and to $\alpha_3\beta_5$ and $\alpha_m\beta_5$ integrins are important for high efficiency infection of target cells. The expression of CAR and α_v integrins on test cells was analyzed by flow cytometry using monoclonal antibodies against CAR (RmcB (Bergelson, J. M., et al. 1997. *Science.* 275:1320-1323; Hsu, K.-H., L., et al. 1988. *J. Virology.* 62:1647-1652)) and α_v integrins (L230 (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621)) (Figure 10). As expected, nearly all HeLa cells expressed high levels of CAR and α_v-integrins, whereas CHO cells lacked significant CAR and α_v-integrin expression. Fifteen and 77% of K562 cells expressed CAR and α_v-integrins, respectively. Only ~6% of the CD34+ cells used in our studies expressed CAR and 17% were positive for α_v-integrins. Notably, the preparation of CD34+ cells represents a mixture of different cell types. The absent or low expression of primary and secondary Ad5 receptors on non-cycling human CD34+ cells is in agreement with previous reports (Huang, S., et al. 1996. *J. Virology.* 70:4502-4508; Neering, S. J., et al. 1996.. *Blood.* 88:1147-1155; Tomko, R. P., et al.. 1997. *Proc. Natl. Acad. Sci. USA.* 94:3352-3356).

Infection assay using wild-type Ad5 and K562 cells:

The presence of viral DNA in the nucleus of infected cells is an indirect means to demonstrate efficient virus binding, internalization, and nuclear import. Nuclear

localization of the viral genome is a prerequisite for transgene transcription and integration. Two techniques are utilized to tag viral DNA for in situ analysis. To optimize the infection assay, wild-type Ad5 virus and K562 cells which are permissive for Ad5 infection can be used. The first protocol (Challberg, S.S. and Ketner, S. 1981, *Virology* 114, 196-209), is based on ^{32}P-labeling of viral DNA. During amplification of wild-type Ad5 and A549 cells, ^{32}P-phosphate (40μCi/ml) is added to phosphate-free medium. After development of CPE, ^{32}P-tagged virus is harvested, banded in CsCl gradients, and titered on HeLa cells according to standard protocols. To simulate the conditions for infection of human bone marrow cells, K562 cells are incubated in suspension with a MOI of 1, 10, or 100 of ^{32}P-Ad5 for 2, 4, 6, or 8 hours under agitation at 37°C. This covers the time period necessary for adsorption, internalization, and nuclear import. After washing, cells are fixed either transferred to microscopy slides using cytospin or embedded in paraffin and sectioned (according to protocols from VECTOR labs, Burlingham, CA). The latter has the potential advantage that multiple consecutive sections (5μm) of the same cell can be analyzed by different methods (e.g. for ^{32}P tagged viral DNA, for specific histological staining, for immunofluorescence), which allows for correlating infection with a particular cell type present in the bone marrow. Cells are incubated in a Kodak NTB-2 photo emulsion for autoradiography. The exposure time can be optimized to minimize background or non-nuclear localized signals. A dose and time dependent appearance of nuclear silver grains is expected under the optimized conditions. Since ^{32}P-phosphate can label viral proteins as well, a cytoplasmic background signal might appear. To facilitate detection, immunofluorescence with HSC specific antibodies on sections can be performed. As an alternative method, a BrdU-labeling technique for viral DNA can be used (Lieber, A., et al. 1999. *J Virol* 73:9314-24; Lieber, A. et al., 1996, *Journal of Virology*, 70:8944-60). In this case, different amounts of BrdU are added to the A549 culture medium during wtAd5 virus propagation. BrdU labeled viral DNA can be detected with monoclonal antibodies specific to BrdU. The signal can be enhanced using layers of species-specific polyclonal antibodies in combination with biotin/avidin and a fluorescent marker. BrdU tagged viral DNA can be detected on cytospins of bone marrow cells together with cell surface markers by double or triple immunoflourescence.

DISCUSSION

The interaction of selected Ad serotypes with CD34+ cells was tested. As a result of this screening we constructed a first-generation, Ad5-based vector whose fiber was substituted with the fiber derived from Ad35. We demonstrated that this capsid modification allowed for efficient viral transduction of potential HSCs by the corresponding chimeric Ad vectors.

All tropism and transduction studies were performed with non-cycling CD34+ cells, which are thought to include HSCs. The quiescent stage of CD34+ cells purified from mobilized blood is important because induction of cell proliferation is associated with a loss of the ability to reconstitute hematopoiesis and with changes in the spectrum of cellular receptors (Becker, P. S., et al. 1999. *Exp. Hematol.* 27:533-541). It is known that treatment of hematopoietic cells with cytokines or growth factors changes the expression of specific integrins including α_v-integrins, which would ultimately alter the susceptibility of cells to Ad infection or may effect viability of infected cells (Gonzalez, R., et al. 1999. *Gene Therapy.* 6:314-320; Huang, S., et al. 1995. *J. Virology.* 69:2257-2263). Another fact that complicates the interpretation of transduction studies is the extraordinary heterogeneity of CD34+ cells in regards to morphology and function.

B. Screening different adenoviruses to establish tropism to HSC.

The ATCC provides more than 70 different human or animal adenoviruses (see Appendix I). A collection of 15 human serotypes and 6 animal adenoviruses (see Table II) are selected based on the following criteria: (i) availability of the complete genome sequence or fiber sequence from the NIH gene bank (ii) CAR receptor usage absent or unknown, (iii) different subgroups, and (iv) moderate or low tumorigenicity (Shenk, T., 1996, *In* B. N. Fields, et al. (eds.), *Fields Virology,* vol. 2 Lippincott-Raven Publisher, Philadelphia). However, any serotype shown in the Appendix hereto can be used for the invention described. Animal viruses are included in the infectivity assay because this may provide a

means to circumvent the pre-existing humoral immunity against human Ad5 fiber, which represents a critical obstacle for clinical trials with Ad vectors.

METHODS

Viruses:

The following human adenovirus serotypes were purchased from the ATCC: 3 (VR-3), 4 (VR1081), 5 (VR-5), 9 (VR1086), 35 (VR-716) and 41(VR-930). Adenovirus No. VR-716 was purchased from ATCC labeled as serotype 34, however it was found to be serotype 35 upon sequencing of the fiber region. For amplification, the corresponding Ads were infected onto HeLa, 293, or HEp-2 cells under conditions that prevented cross-contamination. Virus was banded in CsCl gradients, dialyzed and stored in aliquots as described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaque titering was performed as follows: Confluent 293 cells plated in 6-well plates were incubated for 24 hours with virus in a total volume of 1ml. Two weeks after infection, plaques were counted on cultures overlayed with 1% agarose/MEM/10% FCS.0

EM studies:

CsCl-banded Ad stocks were thawed and diluted with 0.5% glutaraldehyde. Grids were prepared as described earlier (Mittereder, N., et al. 1996. *J. Virology*. 70:7498-7509). After staining with 2% methylamine tungstate (Nanoprobes, Stony Brook, NY), the carbon-coated grids were evaluated and photomicrographed with a Phillips 410 electron microscope, operated at 80 kV (final magnification 85,000x). For each particular Ad serotype, the number of morphologically deficient viral particles per 100 was counted in five random fields.

RESULTS

Electron microscopy:

Little is known about the stability of particles from serotypes other than Ad5. Since the intactness of viral particles was crucial for comparative interaction studies, virions from the serotypes specified above were analyzed by electron microscopy (EM). EM studies of negative contrast stained Ad suspensions demonstrated that the percentage of defective particles (loss of icosahedral shape or luminal staining) did not exceed 5% indicating that serotype preparations had comparable qualities. Representative EM photographs are shown for Ads 5, 9, and 35 (Figure 11).

Serotype screening:

It is thought that different Ad serotypes bind to different cellular receptor proteins and use different entry mechanisms (Defer, C., et al., P. 1990. *J. Virology.* 64:3661-3673; Mathias, P., et al. 1994. *Journal of Virology.* 68:6811-6814). A set of human adenoviruses was obtained from the ATCC to be tested for tropism to CD34+ cells. These included serotypes 3, 4, 5, 9, 35, and 41 representing different subtypes (Table 1). We believed that these serotypes would use different cellular attachment and internalization strategies due to differing lengths of fiber shafts (Chroboczek, J., et al. 1995. *Adenovirus fiber*, p. 163-200. *In* a. P. B. W. Doerfler (ed.), *The molecular repertoire of adenoviruses*, vol. 1. Springer Verlag, Berlin; Roelvink, P. W., et al. 1998. *J. Virology.* 72:7909-7915), the presence or absence of RGD motifs within the penton base, and differing tissue tropism. The relatively little characterized Ad35 was selected because it was found in immunocompromised hosts, particularly in bone marrow recipients (Flomenberg, P., et al. 1994. *Journal of Infectious Diseases.* 169:775-781; Flomenberg, P. R., et al. 1987. *Journal of Infectious Diseases.* 155:1127-1134; Shields, A. F., et al. 1985 New England *Journal of Medicine.* 312:529-533). The latter observations prompted us to believe that bone marrow cells are among the natural reservoirs for Ad35.

TABLE II

Human and animal adenoviruses with potential interest for the invention

Adeno virus	Human/ Group B	Human/ Group D	Human/ Group F	Avian	Bovine	Canine	Ovine	Swine	Mouse
Sero type	3,7,11,16, 21,34,35	8,15,17, 19,28,37	40, 41	CELO, EDS	3	1,2	5	4	1

The underlined serotypes use CAR independent pathways for cell entry.

For amplification, the corresponding adenovirus stocks can be infected onto HeLa or A549 cells such that at a given time only one virus type is handled in a separate laminar flow hood and cultured in Hepa-filtered bottles, preferentially in separate CO_2 incubators to avoid cross-contamination. During propagation, viral DNA is tagged using one of the techniques described earlier. Viral DNA can be isolated from purified particles. The XhoI restriction pattern is analyzed for methylated and unmethylated viral DNA by Southern blot using the full genome of the corresponding virus type as a radioactive probe.

DISCUSSION

Although it was reported earlier by slot-blot assay that fiber knobs derived from 2, 9, 4, and 41L can bind to CAR (Roelvink, P. W., et al.. 1998. *J. Virology*. 72:7909-7915), it is not clear whether this binding occurs with an affinity that is physiologically relevant and whether this would confer cell entry. Furthermore, as shown for the Ad5 interaction between the penton and intergrins, a secondary receptor is required to induce virus internalization. We demonstrated that different serotypes interacted differently with the K562 or CD34+ target cells. Ad5, Ad4, and Ad41 were not able to efficiently attach to and be internalized by K562 and CD34+ cells. Although Ad4 belongs to a separate subgroup (E), it is thought that Ad4 represents a natural hybrid between subgroup B and C viruses with a fiber related to Ad5 (Gruber, W. C., et al. 1993. *Virology*. 196:603-611).

Therefore, it was not surprising that Ad4 has binding properties similar to Ad5. The subgroup F serotype Ad41 has been shown to contain distinct fibers, a long shafted and a short-shafted fiber allowing for different cell entry pathways (Tiemessen, C. T., Kidd, A.H. 1995. *J. Gen. Virol.* 76:481-497). The Ad41 penton base does not contain RGD motifs suggesting that this virus may use α_v-intregrin independent pathways for cell entry. However, these features did not improve interaction with CD34+ cells. Ad9, Ad3, and Ad35 did interact with CD34+ cells more efficiently than Ad5. Out of all the serotypes tested, Ad35 demonstrated the most efficient attachment and internalization with K562 and CD34+ cells. Although the short-shafted Ad9 can bind to CAR, it preferentially uses α_v-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Therefore, the low level of α_v-integrin expression on certain subset/s of CD34+ cells may account for the observed susceptibility to Ad9.

<u>C. Attachment and Internalization of the Ad serotypes to K562 and CD34+ cells.</u>

METHODS

<u>Labeling of Ads with [^{3}H]-methyl thymidine:</u>

Serotypes were labeled with [^{3}H]-methyl thymidine as described in detail elsewhere (Roelvink, P. W., et al. 1996. *J. Virology.* 70:7614-7621). Briefly, 5×10^7 HeLa or 293 cells were grown in 175 sq. cm flasks with 15 ml DMEM/10% FCS and infected with wild type adenovirus at a MOI of 50 or higher. Twelve hours post-infection, 1 mCi of [^{3}H]-methyl thymidine (Amersham, Arlington Heights, IL) was added to the media and cells were further incubated at 37°C until complete CPE was observed. Then, cells were harvested, pelleted, washed once with cold PBS, and resuspended in 5 ml PBS. Virus was released from the cells by four freeze-thaw cycles. Cell debris was removed by centrifugation and viral material was subjected to ultracentrifugation in CsCl gradients and subsequent dialysis as previously described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology.* 70:8944-8960). Virus purification and dialysis removed unincorporated radioactivity. Wild type Ad particle concentrations were determined

spectrophotometrically by measuring the OD_{260}, utilizing the extinction coefficient for wild-type Ad5 $\varepsilon_{260} = 9.09 \times 10^{-13}$ OD ml cm $virion^{-1}$ (Maizel, J. V., et al. 1968. *Virology*. 36:115-125). The virion specific radioactivity was measured by a liquid scintillation counter and was always in the range of 1×10^{-5} to 1×10^{-4} cpm per virion. For selected variants, the fiber gene was PCR amplified and sequenced to ensure identity and the absence of cross-contamination.

Viral DNA tagged with methylase and test for replication by genomic Southern blots:

To ultimately confirm transduction, a protocol to detect adenoviral replication in infected cells can be established. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. A site-specific methylation strategy is utilized to monitor viral DNA replication within infected cells (Nelson, J. et al., 1997, *Journal of Virology*, 71:8902-07). Methylation marked adenovirus can be produced by the addition of a methyl group onto the N6 position of the adenine base of XhoI sites, CTCGAG, by propagation of the virus in HeLa or A549 cells expressing the XhoI isoschizomer PaeR7 methyltransferase (PMT) (Kwoh, T.J., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 7713-7717). It is known that methylation does not affect vector production but does prevent cleavage by XhoI. Loss of methylation through viral replication restores XhoI cleavage and can be detected by Southern blots of genomic DNA from infected cells in comparison to native, non-methylated, viral genomes.

Attachment and internalization assays:

These studies were performed based on a protocol published elsewhere (Wickham, T. J., et al. 1993. *Cell*. 73:309-319). In preliminary experiments, we found that labeled Ad5 virions reached equilibrium in attachment to HeLa cells after 45 min at 4°C with an MOI of 400 pfu per cell. For attachment studies, 3.5×10^5 cells were incubated for one hour on ice with equal amounts of [^{3}H]-labeled adenovirus OD particles equivalent to an MOI of 400 pfu/cell for Ad5 in 100 µl of ice-cold adhesion buffer (Dulbeco's modified Eagle's medium supplemented with 2 mM $MgCl_2$, 1% BSA, and 20 mM HEPES). Next, the cells

were pelleted by centrifugation for 4 min at 1000 x g and washed two times with 0.5 ml ice-cold PBS. After the last wash, the cells were pelleted at 1500 x g, the supernatant was removed, and the cell-associated radioactivity was determined by a scintillation counter. The number of viral particles bound per cell was calculated using the virion specific radioactivity and the number of cells. To determine the fraction of internalized [^{3}H]-labeled adenoviral particles, cells were incubated on ice for one hour with the corresponding virus, washed with PBS as described above, resuspended in 100 µl adhesion buffer, and then incubated at 37°C for 30 min. Following this incubation, cells were diluted 3-fold with cold 0.05% trypsin-0.5mM EDTA solution and incubated at 37°C for an additional 5-10 min. This treatment removed 99% of attached radioactivity. Finally, the cells were pelleted at 1500 x g for 5 min, the supernatant was removed, and the protease-resistant counts per minute were measured. This protocol minimizes the possibility that the internalization data were affected by receptor recycling (Rodriguez, E., Everitt, E. 1999. *Arch. Virol.* 144:787-795). Nonspecific binding of Ad particles to cells on ice was determined in the presence of 100-fold excess of unlabeled virus. This value routinely represented less than 0.1 % of viral load.

RESULTS

Attachment of Ad particles to target cells and internalization:

The selected serotypes were metabolically labeled with [^{3}H]-thymidine, which is incorporated into viral DNA during replication. Adsorption and internalization can be experimentally dissociated by taking advantage of the observation that at low temperature (0-4°C) only virus cell attachment occurs, whereas internalization requires incubation at higher temperatures. The number of particles adsorbed or internalized per cell was calculated using the virion-specific radioactivity and used to quantify interaction of Ads 3, 4, 5, 9, 35, and 41 with CD34+, K562, HeLa and CHO cells (Figure 12). The serotypes varied significantly in their ability to attach to and to be internalized by the different cell lines. For Ad5, the degree of attachment to the cell lines tested correlated with the level of CAR expression. In CHO cells, which were previously shown to be refractory to Ad5

infection, the level of attachment and internalization was about 50-70 viral particles per cell. This number was hereafter assumed negative in terms of susceptibility of a given cell type for Ad5. Interaction of the other serotypes with CHO cells was not significantly higher indicating that corresponding receptor/s were absent on CHO cells. All serotypes tested interacted with HeLa cells; with Ad3 and Ad35 being the most efficient variants. The presence of distinct Ad3 and Ad5 receptors on HeLa cells was demonstrated previously (Stevenson, S. C., et al. 1995. *J. Virology.* 69:2850-2857). Ads 4, 5, and 41 did not bind to K562 cells. In contrast, Ad9 as well as the members of subgroup B, Ad3 and Ad35, efficiently interacted with K562 cells with Ad35 having the highest number of adsorbed and internalized particles. Compared to Ad5, about 25 times more Ad35 particles were attached and three-forth of these were internalized by K562 cells. Viral interactions with CD34+ cells were generally weaker. Among the serotypes tested, only Ad9 and Ad35 were significantly internalized by non-cycling CD34+ cells. Internalization of Ad9 and Ad35 was, respectively, four and eight times more efficient than for Ad5 particles. The number of Ad35 virions internalized by CD34+ cells was almost half of that seen for Ad5 in HeLa cells, which can be readily infected with Ad5 based vectors.

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into HeLa, 293, and CHO cells:

Hela and 293 cells expressing high level of primary and secondary receptors for human adenoviruses are used as a positive control for virus attachment and internalization. As a negative control CHO cells are used. CHO cells do not express the primary adenoviral receptor at a detectable level, and are therefore refractory for adenoviral infection. For attachment studies, these adherent cell lines are detached from 10 cm dishes with PBS-EDTA solution (without Ca2+ and Mg2+), washed three times with ice-cold PBS, resuspended in adhesion buffer, and incubated with viruses as described above in the Examples section. As expected, all adenoviral serotypes tested are efficiently attached to and internalized into Hela cells (Table III) (Figure 13). Adenoviruses serotypes 3, 5, 35, 41, but not 9, are efficiently attached to and internalized by 293 cells. In contrast, poor

attachment and internalization of most adenovirus serotypes are observed with CHO cells. The level of attachment on CHO is about 50-70 virus particles per cell for adenoviruses serotypes 5 and 41, 115 virus particles per cell for adenovirus type 3 and about 180 particles per cell for adenovirus serotypes 9 and 35. For further analysis, numbers >300 viral particles per cell are assumed as positive and <70 viral particles per cell as negative in terms of susceptibility of a particular cell line for efficient adenoviral transduction.

TABLE III

Comparative analysis of attachment and internalization of Ad5 and Ad9 to cell lines, expressing different amounts of CAR and αυβintegrins.

Cell line	CAR expression	αυβ-integrin expression	Ad9 (attached/ internalized)	Ad5 (attached/ internalized)
HeLa	++	++	426/370	550/500
CHO	-	++	300/300	70/50
293	++	++	20/20	1950/1750
Y79	+++	-	190/140	1200/1100
K562	-	+	320/230	60/50
Erythrocytes	?	?	420/-	68/-

Attachment and internalization of adenovirus serotypes 3, 5, 9, 35 and 41 into human CD34+ bone marrow cells and K562 erythroleukemia cell line:

Previous studies showed that the human erythroleukemia cell line K562 can be transduced with Ad5-based adenoviral vectors at very high MOIs. As shown in Figure 14, only about 60 viral particles per cell of adenovirus serotype 5 are attached to and even fewer particles are internalized into these cells at a MOI of 400. In contrast to Ad5, about 320 viral particles per cell of Ad9 and about 1500 viral particles per cell of Ad35 are

attached to and about two-thirds of them are internalized into K562 cells (Figure 14B). Human unstimulated CD34+-enriched bone marrow cells obtained from frozen stocks are incubated overnight in growth medium without cytokine stimulation. The next day, the number of viable cells is calculated. For attachment studies, cells are washed three times with ice-cold PBS, resuspended in adhesion buffer and incubated with adenoviruses. Among the adenoviral serotypes tested, only adenovirus particle of Ad9 (about 150 viral particles per cell) and Ad35 (about 320 viral particles per cell) are able to attach to unstimulated CD34+ cells on the level, compared to Ad 5 (only 60 viral particles per cell). Four-fifths of these virus particles are able to be internalized by the cells. Interestingly, upon stimulation of CD34+ cells with GM-CSF and EPO/TPO for two weeks, attachment and internalization of Ad9 viral particles are significantly increased (up to 300 particles per cell). At the same time, the transient stimulation of cells with GM-CSF for two days could not increase the level of viral attachment to the cells.

Based on the above finding that Ad35 serotype is able to attach and internalize into CD34+ cells most efficiently among several serotypes tested, serotype Ad35 was selected for further studies. As described in Appendix II, a chimeric vector (Ad5 GFP/F35) containing the short-shafted Ad35 fiber sequence in an Ad5 capsid was able to target a broad spectrum of CD34+ cells in a CAR/integrin independent manner.

DISCUSSION

In summary, from all the serotypes tested, Ad9, Ad3, and Ad35 demonstrated the most efficient attachment to and internalization with K562 and CD34+ cells. Based on adsorption/internalization data, Ad9 and Ad35 as representatives for subgroups D and B were selected for further tropism studies.

<u>D. Characterization of Ad vector replication in K562 and CD34+ cells.</u>

Comparative analysis of Ad5, and Ad9 and Ad34 to infect and to replicate in 293, K562 and CD34+ cells. The ability of the Ad9 fiber knob domain to recognize the same

primary receptor on the cell surface as Ad5 with comparable affinity was described earlier. Thus, the finding that Ad9 viral particles can only poorly attach to 293 cells is rather unexpected. In order to find out how the attachment and internalization data reflect the biological activity of adenoviruses of different serotypes, the stocks of Ad5, Ad9 and Ad35 are characterized in more detail by electron microscopy, plaque assay on 293 cells, and quantitative replication assay in K562 and CD34+ cells.

METHODS

Quantitative replication assay:

$1x10^5$ CD34+ or K562 cells were infected in 100μl of growth media with different MOIs of Ad5, 9, or 35 which had been amplified in 293 cells, expressing the XhoI DNA methyltransferase isoshizomer PaeR7 (Nelson, J., Kay, M.A. 1997. *Journal of Virology.* 71:8902-8907). After 2 hours of incubation at 37°C, the cells were centrifuged at 1000 x g for 5 min, the virus-containing medium was removed, the cells were resuspended in 100μl of fresh media, and then they were incubated at 37°C until harvesting. At 16 hours post-infection for K562 cells, or 36 h post-infection for CD34+ cells, 5 μg of pBS (Stratagene, La Jolla, CA) plasmid DNA was added as a carrier which could also be used as a loading control. Genomic DNA was extracted as described previously (Lieber, A., C.-Y. et al. 1996. *Journal of Virology.* 70:8944-8960). One-fourth of purified cellular DNA (equivalent to 2.5×10^4 cells) was digested with HindIII, XhoI, or with HindIII and XhoI together at 37°C overnight and subsequently separated in a 1% agarose gel followed by Southern blot with chimeric Ad5/9 or Ad5/35 DNA probes. The chimeric probes, containing sequences of Ad5 and Ad9 (Ad 5/9) or Ad5 and Ad35 (Ad 5/35), were generated by a two-step PCR amplification using Pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA) and viral DNA from purified particles as a template. The following primers were used for PCR (Ad5 sequences and nucleotide numbers are underlined): Ad5F1 – <u>(nt: 32775-32805)</u> 5'-<u>GCC CAA GAA TAA AGA ATC GTT TGT GTT ATG</u>-3'; Ad5R1 – <u>(nt: 33651-33621)</u> 5'-<u>AGC TGG TCT AGA ATG GTG GTG GAT GGC GCC A</u>-3'; chimeric Ad5/9F – (nt: <u>31150-31177</u>, nt: 181-208) 5'-<u>AAT</u>

GGG TTT CAA GAG AGT CCC CCT GGA GTC CTG TCA CTC AAA CTA GCT GAC CCA -3'; chimeric Ad5/9R – (nt: 32805-32775, nt:1149-1113) 5'- CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC TTC ATT CTT GGG CGA TAT AGG AAA AGG-3; chimeric Ad5/35F – (nt: 31150-31177, nt: 132-159) 5'-AAT GGG TTT CAA GAG AGT CCC CCT GGA GTT CTT ACT TTA AAA TGT TTA ACC CCA-3', chimeric Ad5/35R (nt: 32805-32775, nt: 991-958) 5'-CAT AAC ACA AAC GAT TCT TTA TTC TTG GGC ATT TTA GTT GTC GTC TTC TGT AAT GTA AG-3'. Nucleotide numbers are given according to the sequences obtained from the NCBI GenBank (accession No. M73260 / M29978 for Ad5, X74659 for Ad9, and U10272 for Ad35). After the first amplification, the 968 bp-long Ad9, a 859 bp-long Ad35 DNA fragments corresponding to the fiber genes, and a 876 bp-long Ad5 fragment corresponding to the Ad5 E4 region (located immediately downstream of Ad5 fiber gene) were purified by agarose gel electrophoresis. To generate chimeric DNA probes, amplified Ad5 DNA was mixed with Ad9 or Ad35 fragments obtained during the first step of PCR, and subjected to a second PCR amplification using Ad5/9F or Ad5/35F primers and the Ad5R1 primer. The resulting Ad5/9 or Ad5/35 chimeric DNA fragments (see Fig. 15) were purified and their concentrations were measured spectrophotometrically. Corresponding chimeric DNA fragments were loaded as concentration standards on agarose gels or labeled with [^{32}P]-dCTP and used as probes for Southern analysis. The number of viral genomes per DNA sample was calculated after quantitative Phospho-imager analysis. In preliminary experiments, no preferential hybridization of chimeric DNA probes to DNA of any particular viral serotype was detected.

RESULTS

Replication of selected serotypes in K562 and CD34+ cells:

Adsorption/internalization studies do not ultimately prove viral transduction, a process often defined as gene transfer that allows for viral or heterologous gene expression in host cells. Intracellular trafficking, including endosomal lysis, transport to the nucleus,

and nuclear import of the viral genome, depends on structural capsid proteins and thus, varies between different serotypes (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Miyazawa, et al. 1999. *J. Virology*. 73:6056-6065). We believed that analysis of viral gene expression would be a means to verify successful nuclear import of viral genomes and that this would be a good criterion for selection of serotype/s able to efficiently infect our target cells. To do this, we used a protocol, which allows for the detection of Ad replication in infected cells. Viral DNA synthesis can only occur after de novo expression of adenoviral early genes. We utilized a site-specific methylation strategy to monitor viral DNA replication within infected cells (Nelson, J., Kay, M.A. 1997. *Journal of Virology*. 71:8902-8907). Methylated Ad serotypes were produced by the addition of a methyl group onto the N6 position of the adenine base of Xho I sites, CTCGAG, during propagation of the viruses in 293 cells expressing the Xho I isoschizomer PaeR 7 methyltransferase (PMT) (Kwoh, T. J., et al. 1986. *Proc.Natl.Acad.Sci. USA*. 83:7713-7717) (293 PMTcells). Loss of methylation through viral replication restores Xho I cleavage and can be detected by Southern blots of Xho I-digested genomic DNA from infected cells.

Ad replication studies were performed in K562 and CD34+ cells with Ad9 and Ad35, in comparison to Ad5. For replication studies, the infectious titer (in pfu/ml) and genome titer (in genomes per ml) were determined (by plaque assay on 293 cells or by quantitative Southern blot, respectively) for methylated and unmethylated Ad5, Ad9, and Ad35 (Table 2). The ratio of pfu to genome titer was comparable for methylated and unmethylated virus demonstrating that DNA methylation had not altered transduction properties. About 85% of (Ad5, 9, and 35) virus used for infection was methylated as calculated based on the intensity of fragments specific for methylated and non-methylated viral DNA present in the viral load (Fig. 15). The numbers of genomes detected after adsorption (1 hour, 0°C) or internalization (2 hours 37°C) correlated well with studies shown in Fig. 12. Ad9 and Ad35 interacted more efficiently than Ad5 with K562 and CD34+ cells. Dose-dependent replication studies in K562 and CD34+ cells were performed with the same genome numbers of Ad5, 9, and 35 (Fig. 15). The replication rate was measured based on the ratio of methylated to demethylated viral

DNA after infection with different MOIs (2100, 420, and 105 genomes per cell). In K562 cells, efficient replication (100% conversion from methylated to unmethylated DNA) was detected for Ad5 at MOI >/= 2100, for Ad9 at MOI >/=420, and for Ad35 at MOI >/=105. This demonstrated that Ad35 transduced K562 cells with the highest efficiency. In CD34+ cells, the replication rate was 100% for Ad5 and 31% for Ad9 after infection with MOI 420. Although methylated Ad35 viral DNA was present in CD34+ cells, viral replication was undetectable for Ad35. In summary, while viral replication studies in K562 cells confirmed data obtained for Ad5, 9, and 35 adsorption and internalization, there was a discrepancy between earlier results and the poor replication of Ad9 and, particularly, Ad35 in CD34+ cells. As outlined later, replication analysis in heterogeneous cell populations, like CD34+ cells, may not allow for definitive conclusions on tropism of a particular serotype.

Taking all the screening data together, Ad9 and Ad35 emerged as the variants with the strongest tropism for K562 and CD34+ cells. It is thought that Ad9 can bind to CAR, however, it preferentially uses α_V-integrins for cell entry (Roelvink, P. W., et al. 1996. *J. Virology*. 70:7614-7621). This entry strategy may be not optimal for efficient infection of CD34+ cells as only less that 17% of them express α_V-integrins (Fig. 10). Therefore, we decided to concentrate on Ad35 as a source for heterologous fiber to be used for construction of a chimeric vector based on an Ad5 backbone.

TABLE IV

Results from the infectivity assay which determines the optical particle-to-PFU (OPU/PFU) ratio using 293 cells

Virus	OPU (A260)	PFU	OPU/PFU ratio
Ad5	1.4×10^{12}	1.06×10^{11}	13
Ad9	4.61×10^{11}	2.6×10^{8}	1773

DISCUSSION

Viral replication studies in K562 cells confirmed the data obtained for Ad5, 9, and 35 adsorption and internalization. However, there was a discrepancy between the interaction data and the replication data in CD34+ cells where Ad9 replicated only poorly and no replication was seen for Ad35. Ad replication is only initiated upon the production of a critical threshold of early viral proteins, which in turn, is directly dependent on the number of viral genomes present in the nuclei of infected cells. Therefore, the outcome of replication studies may be affected by the rate of nuclear import of viral genomes, by the activity of viral promoters, and/or the intracellular stability of viral DNA/RNA. These parameters may vary, on one hand, between different subsets of CD34+, and/or, on the other hand, between different Ad serotypes. In conclusion, the viral replication analyses performed with different Ad serotypes in CD34+ cells may not predict the actual transduction properties of chimeric vectors based on Ad5 backbone. This implies that attempts to produce gene transfer vectors based on Ad genomes other than Ad5 should be exercised with caution.

Recently, an Ad serotype screening strategy was used to identify variants with tropism for primary fetal rat CNS cortex cells or human umbilical vein endothelial cells. The optimal serotype (Ad17) was selected based on immunohistochemistry for hexon production 48 hours after infection (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540). However, this approach is problematic because, at least in our hands, antibodies developed against Ad5 hexon did not cross-react with other serotypes. Also, hexon is expressed only after onset of replication. As outlined above, the kinetics of intracellular trafficking, viral gene expression, and replication significantly vary between serotypes (Defer, C., et al., P. 1990. *J. Virology*. 64:3661-3673; Miyazawa, et al. 1999. *J. Virology*. 73:6056-6065).

In addition to being the most efficient serotype in terms of interaction with CD34+ cells, Ad35 is also interesting because it interacts with receptor/s different from the Ad5 and Ad3. Ad35 and Ad5GFP/F35 attachment was not inhibited by Ad5 or anti-CAR antibodies suggesting that Ad35 binding was CAR independent. First, Ad5 did not compete with Ad35 and Ad5GFP/F35 during internalization and infection indicating that

$\alpha_{III}\beta_{3/5}$- integrins are not involved in viral entry. Second, function-blocking antibodies against α_v-integrins did not compete with Ad35 and Ad5GFP/F35 for internalization into K562 cells, whereas these antibodies did inhibit Ad5 internalization. And third, in contrast to Ad5 based vectors, GFP expression after infection with Ad5GFP/F35 was not restricted to α_v–integrin-expressing CD34+ cells. From these facts, we conclude that infection with Ad35 and the chimeric Ad5GFP/F35 vector does not involve α_v-integrins. In this context, the presence or absence of RGD motifs within Ad35 penton base remains to be determined by sequencing the corresponding genome region. Cross-competition assays demonstrated that Ad35 and Ad5GFP/F35 bind to a receptor that is different from the Ad3 receptor. Although Ad3 and 35 belong to the same subgroup, they have been divided into two DNA homology clusters, B1 and B2; the amino acids composing their fibers are only 60% homologous. Furthermore, the target tissues for both viruses are different; Ad3 can cause acute respiratory infections, whereas Ad35 is associated with kidney infection (Horwitz, M. S. 1996. *Adenoviruses,* p. 2149-2171. *In* B. N. Fields, Knipe, D.M., Howley, P.M. (ed.), *Virology*, vol. 2. Lippincott-Raven Publishers Inc., Philadelphia). Therefore, it was not surprising to see that Ad3 and Ad35 recognize different receptors.

In conclusion, Ad35 and the chimeric vector enter the cells by a CAR- and α_v-integrin independent pathway. We believe that Ad35 and the chimeric vector binds primarily to its fiber receptor and that this interaction is sufficient to trigger internalization. On the other hand, Ad35 internalization may involve cellular proteins other than α_v-integrins. These membrane proteins can overlap with those for Ad3 internalization and represent β2 integrins, which protrude more from the cell surface than α_v-integrins (Huang, S., et al. 1996. *J. Virology*. 70:4502-4508).

According to EM studies of negative contrast-stained adenoviral suspensions, the percentage of deficient particles for all adenoviral serotypes tested does not exceed 5%. However, plaque assays reveal that the ability to form plaques in 293 cells is significantly different for tested serotypes. The optical particle-to-PFU (OPU/PFU) ratio obtained is 13 for Ad5, which is in good agreement with the previously estimated ratio for this

adenoviral serotype. Importantly, this ratio is about three times higher for adenovirus serotype 35 and more than 150-fold higher for adenovirus serotype 9. Furthermore, quantitative Southern blot using chimeric Ad5/9 and Ad5/35 DNA probes is used to determine the ratio between the genome and transducing titer. This study confirms the data obtained by plaque assay. Quantitative replication assay of these adenoviruses in K562 and CD34+ cells also confirms the ability of Ad9 and Ad34 to more efficiently attach to these cell types. The replication of viral genomes is observed for Ad9 and Ad34 at lower MOIs of infection, compared to Ad5. In conclusion, the data obtained for different serotypes in attachment and internalization are in good agreement with the infectivity data in target cells.

E. Attachment and internalization of different adenoviral serotypes into primary dendritic cells, JAWSII, MCF-7 and REVC cells.

As a proof of principle, the serotype screening strategy can be employed for other important target cells which are refractory to Ad5 infection.

RESULTS

RECV cells are endothelial cells which have to be targeted for approaches that are aimed to gene therapy of restenosis, atherosclerosis, inflammation etc. MCF-7 cells are breast cancer cells isolated from liver metastases which are important targets for tumor gene therapy. The human adenovirus serotypes 3, 5, 9, 35 and 41 are tested to see whether they can attach to and can be internalized by mouse primary dendritic cells, JAWSII cells, MCF-7-human breast cancer cells and REVC endothelial cells. None of the adenoviral serotype tested can efficiently attach to primary dendritic cells. Adenovirus serotype 3 is able to efficiently attach to REVC endothelial cells (about 400 virus particles per cell are attached and about 300 are internalized). In comparison, only 50 Ad5 particles are able to attach to and even fewer are internalized in these REVC. The human breast cancer cells (MCF-7) are previously shown to be refractory to Ad5

infection at low MOIs. However, Ad3 and more efficiently, Ad35 attach to and internalize into MCF-7 cells.

DISCUSSION

The data presented herein indicate that different human adenovirus serotypes recognize different cellular receptors and can therefore infect cell types that are refractory to Ad5 infection. There are adenoviral serotypes that can more efficiently attach and internalize than Ad5 for human CD34+ cells, REVC, K562 and MCF-7 cells. This finding provides a basis for the construction of chimeric adenoviral vectors which are Ad5 vectors containing receptor ligands derived from other serotypes.

F. Infection studies on primary human bone marrow cells.

Since established erythroleukemic cell lines do not represent an adequate model for the ultimate hematopoietic stem cell that has to be targeted in patients in order to achieve long-term reconstitution with genetically modified cells, normal primary human bone marrow cells are used for the initial infection/retargeting studies.

RESULTS

In a first set of tropism studies with different Ad serotypes, whole bone marrow cell suspensions can be used without preselection. This is advantageous because the tropsim of various adenovirus serotypes or genetically retargeted vectors can be analyzed on a broad spectrum of progenitor subpopulations representing myeloid, erythroid, megakaryocytic, lymphoid, dentritic, and monocytic lineages. For short term (< 5 hours) infection studies, bone marrow suspensions can be cultured in IMDM supplemented with 10% FCS, β-mercaptoethanol, and 10u/ml IL-3 for ensuring cell viability.

Mononucleated cell assays:

Mononucleated bone marrow cells can be incubated with MOI 1, 10, 100, or 1000 pfu/cell of the various adenovirus types for a short time. Paraffin sections or cytospins of infected bone marrow cells can be analyzed for nuclear-localized, labeled viral DNA. BrdU labeling can be visualized by immunoflouresence with anti-BrdU antibodies; ^{32}P-tagged viral DNA can be detected by incubation with photo-emulsion. In addition, the same cell material can be analyzed for morphology after specific histo-staining (e.g. Wright-, Hemo3 staining). If required, commercially available antibodies can be used to specific cell surface markers conjugated directly to different fluorochromes (FITC (green), TRIT., RPE, (red), RPE-Cy5, AMCA (blue)) to completely characterize infected bone marrow subpopulations. Colocalization of BrdU-labeled viral DNA (e.g. as FITC signal) with membrane markers signifying infection of specific cell types can be demonstrated; for example, potential stem cells/early progenitors (CD34$^+$, CD38$^-$), megakaryocytes (CD4la+), eryhthroid cells (glycophorin A+), dentritic cells (CDla+), monocytes (CD14+), or myeloid cells (CD15+), etc. The morphological analysis of infected bone marrow subsets gives a first information whether specific adenovirus serotypes can target primitive cell types.

DISCUSSION

Since the different wild-type adenoviruses do not express a uniform marker gene and do not integrate and since detection of tagged viral DNA cannot be done on live cells, it is not possible, at this point, to characterize infected cells for clonogenic or repopulation capacities. Therefore, adenovirus serotypes for retargeting studies are selected, based on their ability to infect in vitro purified CD34+ cells at low MOIs. This subset of bone marrow cells is known to contain long-term reconstituting cells. Infection studies with different adenovirus serotypes can be repeated on purified CD34+ cells (cultured in IMDM +10% FCS, β-mercaptoethanol, and 10 units/ml IL-3) as described above. Purification of CD34+ cells can be performed by direct immunoadherence on anti-CD34 monoclonal antibody-coated plates or on MiniMacs columns as described by

Papyannopoulou (Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69; Papayannopoulou, T. et al., 1993, *Blood*, 81:229). The purity of isolated CDC34+ cells ranges routinely from 80-95%. Analgous infection studies can be repeated with selected adenovirus types on CD34+/CD38- subsets.

To confirm productive infection purified CD34+ cells can be infected with selected (methylase-tagged) serotypes and analyze viral DNA replication. Cultures of purified human bone marrow CD34+ cells can be used for the transduction and integration studies as a model for HSCs.

It was recently demonstrated that HSC activity does exist in CD34-negative human bone marrow subsets (Bathia, M. et al., 1998, *Nature Medicine*, 4:1038-45; Osawa, M., et al., 1996, *Science*, 273:242-5; Goodell, M. et al., 1997, *Nature Medicine*, 3:1337-45; Zanjani, E. D. et al., 1998, *Exp. Hematology*, 26:353-60). $Lin^{-}CD34^{-}38^{-}$ cells can be tested in the retargeting and transduction studies in combination with repopulation assays in SCID-NOD mice.

G. Cloning and insertion of the fiber gene.

METHODS

PCR-cloning of the corresponding fiber gene and insertion into Ad5 based shuttle plasmids instead of the endogenous AD5 fiber:

One or several adenoviruses with tropism to CD34+ or other HSC containing population is selected for further studies described herein. The complete coding region for fiber varies between 1-2kb, depending on the virus type. The fiber encoding sequences can be obtained by PCR with Pfu polymerase from viral DNA isolated from purified particles of the selected virus types. The corresponding primers can be designed based on the fiber sequences available from the EMBL gene bank. The PCR products are cloned as PacI-BalI fragment into pCD4 (Figure 10), a shuttle vector for recombination of RecA+ E.

coli. In pCD4, the heterologous fiber gene is flanked on both sides with Ad5 sequences, which are homologous to regions directly adjacent to the fiber reading frame in Ad5. As an Ad5 (shuttle vector) derived template for recombination, pCD1, a pBHG 10 (Microbix, Toronto, Canada) derivative can be used. The recombination procedure is performed according to a protocol routinely used for recombinant adenovirus generation (Chartier, C., et al., 1996, *J. of Virology*, 70, 4805-4810). Routinely, 90% of the resulting plasmids are accurately recombined. The junctions between the heterologous fiber (X) and Ad5 sequences can be sequenced to confirm the accuracy of recombination. The resulting plasmid is named pAd5fiberX (pAd5^{fx}). The resulting product is used to generate pAd5^{fx}-based Ad.AAV containing the heterologous fiber gene.

Construction of chimeric Ad vectors:

For transduction studies, two Ad vectors were constructed: Ad5GFP and Ad5GFP/F35, containing a chimeric Ad5/35 fiber gene. Both adenoviral vectors contained a 2.3kb, CMV promoter driven EGFP gene [derived from pEGFP-1, (Clontech, Palo Alto, CA)] inserted into the E3 region of Ad5. The EGFP expression cassette was cloned between Ad5 sequences 25,191-28,191 and 30,818-32,507 into a shuttle plasmid, which contained the E3 deletion described for pBHG10 (Microbix, Toronto, Canada). The resulting plasmid was named pAdGFP. For the chimeric vector, the Ad5 fiber gene in pAdGFP was substituted by an Ad5/35 chimeric fiber gene generated by the two-step PCR protocol outlined above. In the first PCR step, three DNA fragments corresponding to i) the Ad5 fiber 5'-nontranslated region and the first 132 bp of the fiber tail domain (nt 30,818-31,174), ii) the Ad35 shaft and knob domains (nt 132-991), and iii) the Ad5 E4 region including the Ad5 fiber polyadenylation signal (nt 32,775-33,651 were amplified by Pfu-Turbo DNA polymerase. The following primers were used: for the Ad5 tail, Ad5F-2 (nt 30,798-30,825) 5'-CGC GAT ATC GAT TGG ATC CAT TAA CTA-3' and Ad5R-2 (nt 31,174-31,153) 5'-CAG GGG GAC TCT CTT GAA ACC CAT T-3'; for the Ad35 shaft and knob, primers Ad5/35F and Ad5/35R (see above); for the Ad5E4 and polyA, primers Ad5F-1 and Ad5R-1 (see above). After 10 PCR cycles, the products were purified by agarose gel electrophoresis, combined, and then subjected to a second PCR

with primers Ad5F-2 and Ad5R-1. The resulting 2115 bp-long chimeric fiber gene contained the Ad5 tail and the Ad35 shaft and knob domains. This product was used as a substitute for the SalI/XbaI Ad5 fiber gene containing fragment in pAdGFP. The resulting plasmid was named pAdGFP/F35. To generate full-length E1/E3 vector genomes, pAdGFP and pAdGFP/F35 were inserted in pAdHM4 (Mizuguchi, H., Kay, M.A. 1998. *Human Gene Therapy*. 9:2577-2583) by recombination in *E.coli* (Chartier, C., E. et al. 1996. *Journal of Virology*. 70:4805-4810). To do this, the RecA+ *E.coli* strain BJ5183 was co-transformed with pAdHM4 linearized by SrfI mixed with the XbaI fragments containing the GFP genes, the Ad5 or Ad5/35 fiber genes, and the Ad5 homology regions. The resulting recombinants were analyzed by restriction analysis. Correct recombinants were amplified in *E.coli* HB101 and purified by double CsCl gradient banding. The plasmids were named pAd5GFP and pAd5GFP/F35. The correct structure of the Ad5/35 chimeric fiber gene was confirmed by endonuclease digestion and sequencing part of pAd5GFP/F35. To produce the corresponding viruses, pAd5GFP and pAd5GFP/F35 were digested with PacI to release the viral genomes and transfected onto 293 cells as described (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960). Plaques developed 7 to 10 days post-transfection in overlayed cultures. Recombinant viruses were propagated in 293 cells and purified by standard methods described elsewhere (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960).

Hemagglutination assay:

Twenty-five microliters of serial dilutions of Ad5, Ad35, or chimeric Ad5GFP/F35 virions in McIlvaine-NaCl buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 [pH 7.2], diluted 1:50 with 0.87% NaCl) were loaded onto 96 well plates. To each dilution, 25 µl of a 1% suspension of monkey erythrocytes (in McIlvaine-NaCl buffer) was added. The sedimentation pattern was determined after incubation for 1 hour at 37°C. All tests were performed in quadruplicates in at least two independent experiments.

Southern blot:

Extraction of genomic DNA, labeling of DNA fragments and hybridization were performed as described earlier (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960).

RESULTS

Construction/Characterization of chimeric fiber:

Previously, it has been shown that exchanging the fiber knob was sufficient to alter the tropism of chimeric Ad vectors (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540; Krasnykh, V., et al. 1998. *J. Virology*. 72:1844-1852; Stevenson, S. C., et al. 1997. *J. Virology*. 71:4782-4790). As outlined above, the length of the fiber shaft may critically determine the entry strategy of a particular serotype. Therefore, we decided to replace not only the Ad5 fiber knob but also the shaft. The chimeric Ad5/35 fiber contained the Ad5 tail (amino acid: 1-44) necessary for interaction with the Ad5 penton base linked to 279 amino acids from Ad35 including the shaft with 7 β-sheets and the knob (Fig. 16A). The endogenous Ad5 fiber polyA signal was used to terminate transcription of the chimeric fiber RNA. The combination of the Ad5 capsid including the RGD motif containing penton base with a short-shafted fiber could be risky because the natural distance between the fiber knob and the RGD motifs was disturbed. The Ad5 fiber was substituted by the chimeric fiber sequences based on an E1/E3 deleted Ad vector. This vector carried a CMV promoter-GFP reporter gene cassette inserted into the E3 region. The corresponding chimeric virus (Ad5GFP/F35) was produced in 293 cells at a titer of $>2\times10^{12}$ genomes per ml. For comparison, an E1/E3 deleted Ad vector containing the original Ad5 fiber gene and the GFP expression cassette was generated (Ad5GFP). The titer and the ratio of physical to infectious particles was similar between Ad5GFP and Ad5GFP/F35 indicating that the fiber modification did not significantly alter the stability and/or growth properties of the chimeric vector. The correctness of the fiber modification was confirmed by restriction analysis of the Ad5GFP/F35 viral genome followed by

Southern blot hybridization (Fig. 16B), direct sequencing of the fiber-coding region, and a functional test for hemagglutination (HA) of monkey erythrocytes. The agglutination of erythrocytes is fiber knob-mediated; it is known that Ad5 does not agglutinate monkey erythrocytes whereas Ad35 efficiently does (Pring-Akerblom, P., et al. 1998. *J. Virology.* 72:2297-2304). In HA tests, Ad5GFP/F35 agglutinated monkey erythrocytes with the same efficiency as Ad35 at dilutions of up to 1:512. In contrast, no hemagglutination was observed with equivalent Ad5 dilutions. This clearly confirmed the functional activity of the chimeric Ad5/35 fiber incorporated into Ad5 capsid.

Generation of chimeric adenoviral vectors (Ad.AAV^{fx}) with heterologous fiber molecules: Adenoviruses with chimeric Ad5-Ad3 fiber are viable and can be produced at high titers (Krasnykh, V., et al., 1996, *J. of Virology*, 70, 6839-6846; Stevenson, S. C. et al., 1997, *J. Virology*, 71:4782-90). In order to test whether the fiber substitution described herein affects production or stability of adenoviruses, two E1-deleted first-generation, adenoviral vectors are produced with the AAV-□gal cassette in 293 cells using standard protocols. The vector is generated by recombination of pAd.AAV-BG (Fig. 17) with pCD1 (containing the endogenous Ad5 fiber); the other vector (with heterologous fiber) is the recombination product of pAd.AAV βGal and pAd5fiberX (pAd5^{fx}). Virus from single plaques is amplified on 293 cells. The production yield per 293 cell can be determined by plaque-titering of 293 cell Lysates. It is anticipated that the fiber modification will not critically affect the stability of chimeric vectors. Finally, bone marrow cells can be infected with the retargeted vectors. Two days after infection, live-cell cytometry is performed for β-gal expression using as substrate Fluorescein di-□-D-Galactopyranoside (FDG) (Cantwell, M.J. et al., 1996 *Blood* 88, 4676-4683; Neering, S. et al., 1996, *Blood*, 88:1147-55; Fiering, S. N. et al., 1991, *Cytometry*, 12:291; Mohler, W. et al., 1996, *PNAS*, 93:57) and the infected cells are characterized for morphology and surface markers. Before and during infection, bone marrow cells can be cultured in IMDM/FCS supplemented with thrombopoietin (Tpo), which supports the survival of HSC (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). Alternatively, retargeted vectors can be

generated with the AAV-GFP (green fluorescence protein) cassette and perform FACS analysis on transduced cells based on GFP and surface marker expression.

H. Competition studies of chimeric fiber protein Ad5/35.

Competition studies:

Cross-competition studies between Ad5, 35, and Ad5GFP/F35 (Fig. 18) for binding and internalization were performed in order to investigate in more detail the pathways which are used by the chimeric vector to infect target cells. Wild-type Ad35 and the chimeric vector Ad5GFP/F35 could recognize the same primary receptor as they competed with each other for the attachment to K562 cells (Fig. 19A, upper panel). This primary receptor is different from that used by Ad5, since neither Ad5 viral particles nor anti-CAR monoclonal antibodies (Fig. 19B, upper panel) were able to abrogate Ad35 or Ad5GFP/F35 binding. In competition studies for internalization, Ad35 and Ad5GFP/F35 competed with each other with equal efficiency. Ad5 and anti-α_v-integrin monoclonal antibodies (L230) (Figs. 19C, D; lower panel) did not inhibit internalization of Ad35 or the chimeric virus. To consolidate this data, K562 cells were infected with Ad5GFP and Ad5GFP/F35 after prior incubation of cells with anti-CAR or anti-α_v-integrins monoclonal antibodies followed by analysis of GFP-expressing cells. The transduction data mirror the results obtained in adsorption/internalization studies. In summary, this demonstrated that Ad35 and Ad5GFP/F35 use a CAR and α_v-integrin-independent pathway for infection of K562 cells; the structural elements which account for these specific properties are located within the Ad35 fiber and can be transplanted into Ad5 by fiber substitution.

Ad3 can efficiently interact with K562 cells (Fig. 12), although Ad3 and Ad35 belong to the same subgroup (B), the homology between amino acid sequences of their fibers is only about 60%. Therefore, we decided to test whether Ad3 could compete with Ad35 and Ad5GFP/F35 for attachment and internalization (Fig. 20). These studies demonstrated that Ad35 binding was not inhibited by Ad3 indicating the use of different

receptors. Interestingly, Ad3 slightly inhibited attachment of Ad5GFP/F35 (Fig. 20A, left panel). In addition to binding to the receptor common for the Ad35 and Ad5GFP/F35 fiber, the chimeric capsid (e.g. the Ad5 penton RGD motifs) may also interacts with a second cellular receptor that overlaps with elements involved in Ad3 binding. In cross-competition for internalization, pre-incubation of cells at 37°C with Ad35 and with chimeric virus significantly decreased internalization of [^{3}H]-labeled Ad3 (Fig. 20D, right panel). In the reverse experiment, Ad3 as competitor decreased the level of internalization by 30% for both, Ad35 and the chimeric virus (Fig. 20B, right panel). As expected, Ad5 and Ad3 did not compete for adsorption or internalization. As shown before (Fig. 19B), anti-CAR and anti-α_v-integrin antibodies did not block Ad3 interaction with K652 cells. In summary, we concluded that Ad35 and Ad5GFP/F35 bind to receptor/s different from that of Ad3, although they can use common structural elements for internalization, which are different from α_v-integrins.

Infection studies with chimeric virus:

It is established that Ad5GFP/F35 infected K562 cells by a CAR and α_v-independent pathway. It is possible that this property allows for efficient transduction of non-cycling CD34+ cells, which express scarcely CAR and α_v-integrins. To test this, the transduction properties of Ad5GFP and Ad5GFP/F35 vectors were analyzed on CD34+ cells, K562, and HeLa cells. Fig. 21 shows the percentage of transduced, GFP expressing cells depending on the MOI used for infection. Nearly 100% of HeLa cells were transduced with Ad5GFP and Ad5GFP/F35 at MOIs of >/=25. More than 95% of the K562 cells were transduced with Ad5GFP/F35 at MOIs of >/= 100, whereas the transduction rate was significantly lower with Ad5 where it increased with the MOI reaching a plateau at ~70% GFP-positive cells after infection with an MOI of 400. Transduction of CD34+ cells was about three fold more efficient with Ad5GFP/F35 than with Ad5GFP at all MOIs analyzed. Interestingly, at higher MOIs, the transduction rate did not rise proportionally with the viral dose and soon reached a plateau indicating that in both cases only specific subset/s of CD34+ cells were permissive to infection. In order to characterize in more detail these specific, permissive subset/s, additional transduction

studies were performed. First, the percentage of GFP expressing cells was determined in CD34+ fractions that were stained for α_v-integrins or CARs (Fig. 22). The low number of CAR positive CD34+ cells complicated accurate co-labeling studies, and there was no correlation between CAR expression and the proportion of transduced cells among CD34+ cells infected with Ad5GFP or Ad5GFP/F35. Interestingly, for Ad5GFP, 65% of all GFP expressing cells were positive for α_v-integrins, whereas less than 22% of GFP positive cells infected with the chimeric virus stained positive for □$_v$-integrin expression. While only 17% of the whole CD34+ population expressed GFP after Ad5GFP infection, the percentage of GFP–expressing cells in the CD34+/ □$_v$-integrins positive fraction was 50%. This indicates that Ad5GFP vector-mediated GFP expression was preferentially localized to α_v-integrin positive CD34+ subsets, whereas after infection with the Ad5GFP/F35 vector, GFP was expressed in a broader spectrum of CD34+ cells with most of them being α_v-integrin-negative.

Next, transduced cells were simultaneously analyzed for GFP as well as for CD34 and CD117 markers. As mentioned before, only about 90% of all cells used in our analysis were positive for CD34 at the time of infection, hence the multiparameter analysis for CD34 and GFP. A population of CD34+ cells is extraordinarily heterogeneous in morphology and stem cell capacity. The subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (*Ikuta, K., Weissman, I.L. 1992* Proc. Natl. Acad. Sci. USA. *89:1502-1506*; *Simmons, P. J., et al. 1994.* Expl. Hematology. *22:157-165*). Fig. 23 summarizes the analyses of GFP expression in correlation with these specific stem cell markers. While 54% of cells infected with chimeric vector were positive for GFP and CD34+, only 25% of cells infected with Ad5GFP expressed the transgene and CD34+ marker (Fig. 23A, lower panel). More importantly, based on GFP expression, the chimeric virus transduced 80% of c-kit positive cells, whereas the Ad5-based vector transduced only 36% (Fig. 23A, middle panel). In an additional experiment, CD34+ cells were sorted for CD117 expression prior to infection with Ad5GFP or Ad5GFP/F35 and, 24 hours post-infection, GFP expression was analyzed in this specific fraction (Fig. 23B). This analysis revealed that the chimeric vectors transduced 4 fold more CD34+/CD117+ than the Ad5GFP vector.

In conclusion, these results demonstrated that the chimeric Ad5GFP/F35 vector was clearly superior to the Ad5GFP vector in targeting and transduction of CD34+ cells. Furthermore, the data suggest that the spectrum of CD34+ cell subsets permissive for Ad infection was significantly different for the chimeric vector than for the Ad5 vector.

Analysis of viral genomes within CD34+ cells infected with the Ad5 and chimeric vectors:

So far, the transduction rate of CD34+ cells was measured based on GFP expression after infection with Ad5GFP and Ad5GFP/F35. Considering the extraordinary heterogeneity of CD34+ cells in morphological and functional parameters, GFP may not be expressed in all cell types that were efficiently infected. Reasons for this include that the CMV promoter may not be active in all cell types or that the regulation of transgene expression could differ between subsets on a post-transcriptional or post-translational level. To test this, we quantified the number of intracellular (transduced) viral genomes within GFP positive and GFP negative fractions of CD34+ cells infected with Ad5GFP and Ad5GFP/F35. To do this, twenty-four hours after infection, CD34+ cells were sorted for GFP positive and GFP negative fractions, which were subsequently used to isolate genomic DNA together with transduced viral DNA. The number of viral genomes was determined by quantitative Southern blot as described for Fig. 15. Per GFP-positive CD34+ cell, about 270 copies of the Ad5GFP/F35 viral genome were detected. Interestingly, a remarkable 200 copies of the Ad5GFP/F35 viral genome were found per GFP-negative CD34+ cell (Fig. 24A and 25). This demonstrated that not all infected cells expressed GFP and implies that the actual transduction rate was higher than 54% (GFP-positive cells). We concluded that the CMV promoter was not active in all transduced CD34+ subsets. No Ad5GFP vector specific signal was detected within infected CD34+ (GFP positive or negative) fractions by Southern blot which had a detection limit of 14 viral genomes per cell. From this, we can conclude that the vector DNA concentration per transduced cell was at least 20 times higher for Ad5GFP/F35 than for Ad5GFP.

Ad5GFP DNA was only detectable in DNA samples from infected CD34+ cells by Southern blot after prior PCR amplification with vector specific primers (Fig. 24B and 25). This indicates that the replication deficient Ad5 vector is present but at a very low copy number, which may be limited by intracellular genome stability. Using the PCR-Southern detection method, Ad5 vector DNA was also detected in GFP negative cells, supporting that the CMV promoter may not have been the optimal choice for transduction studies. It is notable that studies by others on viral genome analyses after infection of CD34+ cells with Ad5 vectors were performed only after prior PCR amplification (Mitani, K., et al. 1994. *Human Gene Therapy*. 5:941-948; Neering, S. J., et al. 1996.. *Blood*. 88:1147-1155).

DISCUSSION

The chimeric Ad5GFP/F35 vector has binding and internalization properties similar to Ad35. Therefore, the fiber substitution was sufficient to swap cell tropism from Ad5 to Ad35. The Ad5GFP/F35 capsid chimera contained the short-shafted Ad35 fiber incorporated into an Ad5 capsid, instead of the naturally occurring long-shafted Ad5 fiber. During Ad5 infection, interaction between the penton base and intergrins is required to induce viral internalization. For this interaction, the length of fiber shaft and the precise spatial arrangement of knob and RGD motifs are critical for the virus entry strategy. The natural spatial arrangement is disturbed when short-shafted heterologous fibers are inserted into the Ad5 capsid. Interestingly, the Ad5/35 capsid chimera allows for efficient infection, suggesting that the protruding RGD motives in the Ad5 penton base do not affect the interaction with the primary Ad35 receptor. So far, most of the chimeric viruses were generated by substituting only the Ad5 knob while maintaining the long Ad5 fiber shaft (Chillon, M., et al. 1999. *J. Virology*. 73:2537-2540; Krasnykh, V. N., et al. 1996. *J. Virology*. 70:6839-6846; Stevenson, S. C., et al. 1995. *J. Virology*. 69:2850-2857; Stevenson, S. C., et al. 1997. *J. Virology*. 71:4782-4790). The exception was an Ad5/7 chimeric virus (Gall, J., et al. 1996. *J. Virology*. 70:2116-2123), where the whole Ad5 fiber was substituted by the short-shafted Ad7 fiber. However, similar to the parental Ad5, the Ad5/7 chimera still required α_v-integrins for infection.

This Ad5GFP/F35 chimera is the first demonstration that despite the presence of RGD motifs within the Ad5 penton, the chimeric virus uses cell entry pathways determined primarily by the receptor specificity of the short-shafted heterologous fiber. This does not exclude that interaction with a secondary receptor may increase binding affinity. The latter is supported by the observation that Ad35 and Ad5GFP/F35 slightly differed in their ability to compete with Ad5 or Ad3 for binding. It is possible that Ad5/35 attachment involves, in addition to the high affinity fiber binding, interaction between Ad5 capsid proteins (e.g. RGD motifs) and secondary receptor/s that overlap with those used by Ad3 and Ad5.

This data indicate that infection with Ad5-based vectors is restricted to a specific subset of CD34+ cells. The percentage of GFP expressing cells after Ad5GFP infection of CD34+ cells reached a plateau at MOIs higher than 100 indicating that only a limited fraction of CD34+ cells was permissive to Ad5. Also, strong replication of wild type Ad5 in infected CD34+ cells may be the result of preferential transduction of a specific subpopulation of CD34+ resulting in a expression of early viral genes at a level sufficient to initiate viral replication. The presence of a specific subpopulation of CD34+ cells permissive to Ad5-vector infection was suggested by others (Byk, T., et al. 1998. *Human Gene Therapy*. 9:2493-2502; Neering, S. J., et al. 1996.. *Blood*. 88:1147-1155). In the present report, we further characterized this subpopulation and demonstrated that Ad5-based vectors preferentially infected α_v-integrin positive CD34+ cells.. Integrins (includingα_v) are thought to be important for homing and trafficking of transplanted hematopoietic cells, however little is known about the correlation between α_v-integrin expression and the differentiation status of hematopoietic cells (Papayannopoulou, T., Craddock, C. 1997. *Acta Haematol*. 97:97-104; Roy, V., Verfaillie, C.M. 1999. *Exp. Hematol*. 27:302-312). There was no clear correlation between CAR and GFP expression suggesting that Ad5GFP may be able to use another membrane protein as a primary receptor. Alternatively, Ad5GFP transduction observed at an MOI of 200-400 could be the result of direct interaction between virus and α_v-integrins triggering internalization, which may be the preferred pathway in the absence of CAR (Legrand, V., et al. 1999. *J.*

Virology. 73:907-919). Importantly, infection with the chimeric Ad5GFP/F35 vector was not restricted to the α_v-positive CD34+ subpopulation.

Among CD34+ cells, the subpopulation of CD34+ and CD117+ cells resembles very primitive hematopoietic cells (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). The receptor for stem cell factor, CD117 (c-kit) belongs to a tyrosine kinase family. It was previously shown that c-kit+, CD34+ cord blood cells contain a high fraction (16%) of hematopoietic progenitors (Neu, S., et al. 1996. *Leukemia Research*. 20:960-971). Early in ontogeny 34+/CD117+ cells have long-term repopulating activity (Sanchez, M. J., et al. 1996. *Immunity*. 5:513-525). An average of 50-60% of CD34+ cells are reported to be CD117 positive (Ikuta, K., Weissman, I.L. 1992 *Proc. Natl. Acad. Sci. USA*. 89:1502-1506; Neu, S., et al. 1996. *Leukemia Research*. 20:960-971; Simmons, P. J., et al. 1994. *Expl. Hematology*. 22:157-165). In our studies, the chimeric vector expressed GFP in 54% CD34+ cells and 80% of CD34+/c-kit+ cells. The actual viral transduction rate could be even higher because transduced Ad5GFP/F35 vector DNA was also found in GFP-negative fractions of infected cells. This indicates that the CMV promoter used to drive GFP expression in our vectors was not active in all transduced cells. We selected the CMV promoter for transgene expression based on published data demonstrating that PGK and CMV promoters allowed for efficient transgene expression in CD34 cells whereas the HTLV-I and RSV promoter were almost inactive (Byk, T., et al. 1998. *Human Gene Therapy*. 9:2493-2502; Case, S. S., et al. 1999. *Proc. Natl. Acad. Sci. USA*. 96:2988-2993). On the other hand, studies by Watanabe et al. (Watanabe, T., et al. 1996. *Blood*. 87:5032-5039) suggest that the CMV promoter is not active or rapidly silenced in certain CD34+ subsets. Our data underscore this observation. Considering retroviral transduction studies, the retroviral MLV promoter may have been a better candidate for transduction studies in hematopoietic cells (Bregni, M., et al. 1998. *Gene Therapy*. 5:465-472).

After having demonstrated that the Ad5GFP/F35 vector efficiently transduced cells carrying stem cell specific markers, the next logical step would be to perform colony

assays with pre-sorted GFP positive/negative cells. However, this assay is complicated by the fact that infection with first generation Ad vectors is cytotoxic and affects the formation and growth of progenitor colonies in MC-cultures (Mitani, K., et al. 1994. *Human Gene Therapy*. 5:941-948; Watanabe, T., et al. 1996. *Blood*. 87:5032-5039). This side effect is caused by the expression of Ad proteins within transduced cells (Lieber, A., C.-Y. et al. 1996. *Journal of Virology*. 70:8944-8960; Schiedner, G., et al. 1998. *Nature Genetics*. 18:180-183; Yang, Y., et al. 1994. *Proc. Natl. Acad. Sci. USA*. 91:4407-4411). Some of these proteins (e.g E4-orf4, pTP, or E3-11.6k) have pro-apoptotic activity (Langer, S. J., Schaak, J. 1996. *Virology*. 221:172-179; Lieber, A., et al. 1998. *J. Virology*. 72:9267-9277; Shtrichman, R., Kleinberger, T. 1998. *J. Virology*. 72:2975-2983; Tollefson, A. E., A et al. 1996 *J. Virology*. 70:2296-2306). Clearly, this would affect the outcome of transduction studies with Ad5GFP/F35, which allows for the efficient transfer of viral genomes into CD34+ cells implying significant expression of viral proteins. Moreover, recently published data indicate that short-term colony assay mostly measure mature progenitors and do not represent a rigorous test for transduction of potential stem cells.

A definitive demonstration that Ad5GFP/F35 based vectors can transduce HSC requires colony assays or preferably, repopulation assays in SCID-NOD mice. We can perform these studies with gutless vectors (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13) and integrating □Ad.AAV vectors devoid of all viral genes (Lieber, A., et al. 1999. *J Virol* 73:9314-24) generated based on Ad5GFP/F35 chimeric capsids. Alternatively, gutless, retargeted vectors could be used to transiently express a retroviral receptor on CD34+ cells to increase their susceptibility to infection with retroviral vectors based on an approach that we have published earlier (Lieber, A., et al.. 1995. *Human Gene Therapy*. 6:5-11).

Our finding that Ad5GFP/F35 can efficiently transduce hematopoietic cells with potential stem cell capacity represents an important step towards stable gene transfer into HSCs and gene therapy of blood disorders. Furthermore, the virological aspects of this invention contribute to a better understanding of adenovirus cell interactions.

I: Retargeting of Ad5 based vectors with modified fibers carrying specific ligand peptides for HSC and other cell types

Another alternative to make Ad5-capsid-based vectors suitable for HSC gene therapy is to incorporate the coding sequence for HSC specific peptides into the H1 loop region of the Ad5 fiber gene. The modification of the H1-loop was successfully exercised by Krasnykh et al. with a 7 amino-acid long FLAG peptide (DYDDDDK). Using phage display peptide libraries (Pascqualini, R. et al., 1996, *Nature*, 380:364-66), Renata Pasqualini (La Jolla Cancer Research Center) reported recently, at the First Meeting of the American Society for Gene Therapy, the identification of small peptide ligands specific for bone marrow cells. The corresponding sequences encoding these peptides can be added to modify the H1 loop sequence employing site-directed mutagenesis. Optimally, the ligands should allow for the efficient internalization of adenoviral particles based on a CAR- and integrin independent pathway. Modified adenoviral vectors containing the AAVBG cassette can be produced and tested for HSC tropism as described above.

Adenovirus peptide display:

In order to retarget adenoviruses to any cell type of interest, a strategy is provided which involves creating a library of adenoviruses displaying random peptides in their fiber knobs as ligands and screening this library for adenovirus variants with tropism to a particular cell type in vitro and potentially in vivo.

The development of the adenovirus peptide display technique is based on the following ideas. (i) Although the tertiary structure of the Ad5 fiber knob is known, it remains unclear which domains are involved in receptor binding. There are data suggesting that receptor-binding domains partially overlap with hemagglutination domains, which are well characterized for a number of serotypes. Therefore, three intramolecular loop regions representing potential receptor binding sites can be substituted by random peptide

libraries. Eight amino acid residues in the center of the FG, or GH loops can be substituted by octameric random peptides (Figs. 26 and 27). These substitutions will replace CAR tropism and allow for infection of refractory cell types. (ii) To synthesize the oligonucleotides encoding the peptide library a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids is employed. This avoids termination codons and assures optimal codon usage and translation in human cells. Synthesis of a completely randomized library is possible with all 20 amino acids being incorporated with the same probability and a partially randomized library with only three (in average) random amino acids substitutions per octamer at random positions with a random amino acid to maintain certain critical features of the tertiary knob structure while introducing variability. The last model is based on the distribution of amino acids present in the hypervariable CDR 1 or 2 region of immunoglobulins. (iii) To maintain a representative library size of about 10^{10} different octamers per modified loop, a new cloning strategy is employed to allow for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. This strategy is based on a "seamless" cloning technique available from Stratagen. (iv) In order to produce the library of viruses, viral genomic DNA containing the modified fiber sequences is transfected into 293 cells without reduction of the library size. This critical step is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via poly-lysine to assure 100% transfection efficiency. This technique allows for coupling of ~1μg of plasmid DNA (or ~$1x10^{10}$ adenoviral genomes) to 10^{10} viral particles which can be used to infect 293/cre cells at an MOI of 10-100. Importantly, the carrier adenoviral genome has the packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express cre recombinase (293/cre). This helper virus system is routinely used to produce so-called gutless adenoviruses. Therefore, the virus progeny represents library genomes packaged into capsids containing preferentially Ad5 fibers. This is important for the next infection step into 293 cells at a MOI of 1 to assure a homogeneous fiber population on the capsid where the fibers are encoded by the packaged genome.

J. Production Of Adenovirus Vectors With Increased Tropism To Hepatocytes

An example of a G-H loop substitution to target Ad5 to hepacytes was successful. Preliminary tests demonstrated that two evolutionarily conserved regions within the malaria circumsporozoite surface protein (CS) termed RI and RII+ mediate specific interaction with hepatocytes but not with other organs (including spleen, lung, heart and brain), nor with Kupffer cells, liver endothelial cells or with other regions of the hepatocyte membrane (Cerami, C. et al., 1992, *Cell,* 70:1021-33; Shakibaei, M. and U. Frevert, 1996, *J. Exp. Med.,* 184:1699-711). These regions are conserved among different species including Plasmodium berghei, P. cynomogli, and P. falciparum that infect mouse, monkey and human hepatocytes, respectively (Cerami, C. et al., 1992, *Cell,* 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81). Peptides derived from RI (KLKQPG) or RII (EWSPCSVTCGNGIQVRIK) blocked CS binding to hepatocytes and infection by sporozoites in vivo ((Cerami, C. et al., 1992, *Cell,* 70:1021-33; Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81). RI and RII+ peptides were separately inserted into Ad5-fiber knob (H-I and G-H loop) containing mutation with abolished binding to CAR and alpha-v integrins (Kirby, L. et al., 2000, *J. Virol.,* 74:2804-13; Wickham, T. J. et al., 1995, *Gene Ther.,* 2:750-6). Based on preliminary data, a short-shafted fiber was used so that the virus entry strategy predominantly depends on the interaction with the primary (hepatocyte-specific receptor). The hepatocyte-specific ligands are flanked by short glycine stretches to provide flexibility and embedded into a loop formed by two cystines. This is one of the classical strategies to incorporate ligands into a protein scaffold (Doi, N. and H. Yanagawa, 1998, *Cell Mol. Life Sci.,* 54:394-404; Koivunen, E. et al., 1995, *Biotechnology (NY),* 13:265-70) and to guarantee their presentation at the protein surface. The biodistribution of the best variants is tested in vivo in C57B1/6 mice based on Southern blots or PCR for vector DNA in different organs. This mouse strain in known to be susceptible to infection with *P. berghei* (Chatterjee, S. et al., 1995, *Infect Immun.,* 63:4375-81).

K. Production Of Adenovirus Vectors With Increased Tropism To Tumor Cells

A similar strategy is to insert two peptides obtained after selection for tumor tropism by displaying random peptides on filamentous phages. The first double cyclic peptide (RGD-4) proved to bind specifically to integrins present on tumor vasculature (Ellerby H. M. et al., 1999, *Nat. Med.*, 5:1032-8). The second peptide targets specific matrix metalloproteinases associated with metastatic tumor cells as shown for the breast cancer cell line MDA-MB-435 (Koivunen, E. et al., 1999, *Nat. Biotechnol.*, 17:768-74). Tropism-modified vectors are tested in animal models with hepatic metastases derived from MDA-MB-435 cells (Fig. 28).

L. Development Of A Peptide Display Technique Based On Adenoviruses

A synthetic peptide library is described that allows adenovirus vectors to express random peptides in the G-H loop of the fiber knob domain. The technique of a phage display library is optimized to generate a library of adenoviruses displaying random peptides in their fiber knob. This library of adenovirus variants is then screened for tropism to a particular cell type in vitro and potentially in vivo. The oligonucleotides encoding the peptide library employ a novel technique to assemble pre-synthesized trinucleotides representing the codons for all 20 amino acids. This will end the termination codons and assure optimal codon usage and translation in human cells. To maintain a representative library size, a new "seamless" cloning strategy that allows for insertion of the library into the wild-type Ad5 genome without introducing additional amino acids at the substitution site and without transformation into bacteria. Transfection into 293 cells is done by conjugating the viral library DNA to carrier Ad5-based adenovirus via polylysine to assure a 100% transfection efficiency. Importantly, the carrier adenoviral genome has its packaging signal flanked by lox sites preventing the packaging of carrier viral DNA after infection of 293 cells that express Cre recombinase (293/cre). The library is produced with E1-positive viruses depleted for CAR and integrin tropism. Only variant that have successfully infected the cell type of interest will replicate, resulting in de novo produced

virus. The sequence of the peptide ligand that conferred the particular tropism will then be analyzed in do novo produced virus.

EXAMPLE III

COMBINATION NOVEL ADENOVIRAL VECTOR AND MODIFIED FIBER PROTEIN

This example describes the following studies which combine the technology of the integrating adenovirus vector that is devoid of all adenoviral genes with the modified fiber protein that retargets the vector to quiescent HSC.

A. Transduction studies with re-targeted vectors in HSC:

In order to transduce quiescent HSC and integrate into chromosomal DNA, retargeted ΔAd.AAV^{fx} vectors are tested for reporter gene expression, and vector integration simultaneously while analyzing their clonogenic capacity. The modified ΔAd.AAV^{fx} hybrid vectors contain genomes devoid of all adenoviral genes (a "gutless" adenovirus vector) packaged into Ad5 capsids with modified fibers. Rep may be incorporated into these ΔAd.AAV^{fx} vectors to allow for site-specific integration into AAVS1.

Transduction studies:

Purified human CD34+ cells in IMDM/FCS+IL-3 and SCF are infected with different doses of ΔAd.AAV^{fx}-BG (1-10^7 genomes per cell). CD34+ cells infected with ΔAd.AAV^{fx}-βGal are cultured for 2 days in suspension and sort β-Gal+ cells by FACS using FDG as substrate. This determines the infection efficiency. β-gal expressing cells are then submitted to clonogenic assays in semi-solid cultures (in two dishes per MOI) in the presence of multiple cytokines. (IL-3, SCF, Epo, G-CSF, GM-CSF, IL-7, Tpo). A first set of semi-solid cultures can be evaluated after 7 days; another set can be analyzed after 14 days. Colonies that have formed in semisolid culture can be characterized by

light microscopy and subsequently stained with X-Gal staining. Most of the vector genomes should remain episomal and can be lost with successive cell divisions. Thus, while most cells can be X-Gal positive at day 2 or day 7 after infection, most of the larger colonies (analyzed at day 14 p.i.) may not stain homogeneously for β-Gal. A representative number of X-Gal positive and X-Gal negative colonies can be picked and analyzed for episomal and integrated vector DNA. The outcome depends on the MOI used for infection and the integration status of the vector. These studies determine whether hybrid vectors can infect primitive progenitors.

Detailed characterization of hybrid vector integration:

CD34+ cells can be infected with ΔAd.AAV^{fx}-SNori (MOI 1-10^7) and subjected to G418 selection in methyl cellulose (MC) cultures in the presence of growth factors (IL-3 and SCF). The resulting colonies are a mixture of mainly myeloid cells. The number and morphology of G418 resistant colonies can be determined after 2 weeks of selection. This strategy may be disadvantageous in that the appropriate stem cell may not divide and form G418 resistant colonies under the specific culture condition used. Moreover, it may be difficult to perform G418 selection on a population of heterogenous cells, which vary in their sensitivity to G418. Therefore, another set of ΔAd.AAV^{fx}-SNori infected CD34+ cells can be cultured in methyl-cellulose (+IL-3, SCF) without G418 selection. After 2-3 weeks, single colonies can be picked from both (w/ and w/o G418) MC cultures, morphologically characterized, and analyzed for integrated vector using the modified protocol developed for integration studies in a small number of cells (see Figure 8). This strategy allows the assessment of whether hybrid vectors integrate into the genome of CD34+ cells cultured in the presence of growth factors. This study gives us an idea about potential position effects affecting neo or βgal expression from integrated vector copies and about the structure of the integrated vector and the flanking chromosomal regions.

An alternative method to confirm vector integration:

Fluorescence *in situ* hybridization (FISH) analysis, can be performed in individual cells from MC colonies. CD34+ cells are cultured in MC in the presence of growth factors to induce cell division and subsequently treated with colchicine. Metaphase chromosome spreads are analyzed with biotin-ATP labeled probe specific for the βGal or SNori gene and a dioxigenin-UTP labeled probe for the human X-chromosome as an internal control (provided by Christine Disteche, University of Washington). Specific hybridization can be visualized with corresponding anti-biotin or anti-DIG antibodies labeled with different fluorochromes (e.g. FITC and Texas Red). Hybrid vector DNA may integrate as concatemers, which would facilitate detection by FISH. This technique allows one to localize the chromosomal integration sites of hybrid vectors.

Test transduction into quiescent bone marrow subpopulations:

Hybrid vectors described so far can be tested to see whether quiescent CD34+ cells can be stably transduced. To avoid significant cell proliferation, purified CD34+ cells are cultured in serum free IMDM supplemented with thrombopoietin (Tpo). Tpo can alone support the survival of stem cells without stimulating their active cell proliferation (Matsunaga, T. et al., 1998, *Blood*, 92:452-61; Papayannopoulou, T. et al., 1996, *Experimental Hematology*, 24:660-69). To analyze the proliferation status of CD34+ cells at the time point of infection with the hybrid vector ΔAd.AAV^{fx}BG, BrdU is added 2 hours before infection to the culture medium. One set of cells are maintained as suspension culture in IDAM containing Tpo only for two days. Another set of cells are grown in IDAM+Tpo supplemented with multiple cytokines. Forty eight hours after infection, CD34+ cells can be FACS sorted for beta Gal expression using FDG. FDG positive cells can be further analyzed for cellular DNA replication based on BrdU incorporation and for specific CD34+ subset markers. To do this, cytospins from FDG+ cells can be submitted to immunofluorescence with BrdU specific antibodies and with antibodies to specific cell surface markers (e.g. CD38, CD41). Alternatively, consecutive paraffin sections of the same cell can be analyzed for (a) transgene expression by X-Gal staining, (b) DNA synthesis based on BrdU incorporation, and (c) specific surface markers. This allows one to confirm that the culture conditions with Tpo alone prevent

significant genomic DNA replication and subsequent cell proliferation as well as to determine whether quiescent CD34+ cells can be infected based on beta Gal expression in cells where BrdU labeling is absent.

Test hybrid vectors integration into quiescent CD34+ cells:

Two sets of CD34+ cells are infected. The first set of □Ad.AAV^{fx}SNori infected cells are cultured for 5-7 days in the presence of cytokines; the other set is cultured without cytokines. To maintain CD34+ cell viability without cytokines during this period, the cells are cultured in the presence of Tpo or underlaid with a stromal cell line (AFT024) (Moore, K. et al., 1997, *Blood*, 89:4337-47), which can maintain HSC viable for 4 to 7 weeks. After this specific time period, both sets are submitted to clonogenic assays (in the presence of multiple cytokines) either in combination with G418 selection or without selection. Single colonies are analyzed morphologically and submitted to genomic DNA analysis (Figure 8) to determine the vector integration status. The ultimate proof for stem cell transduction is the in vivo survival/expansion assay. To do this, the CD34+ cells expressing beta Gal are used for transplantation experiments, If the number of FDG+ cells is not sufficient, total ΔAd.AAV^{fx}BG infected cells as well as all ΔAd.AAV^{fx}-SNori infected cells can be used directly without selection. Transplantation can be performed via tail vein injection into sublethally irradiated SCID NOD mice (Dao, M.A., et al., 1998, *Blood*, 4, 1243-1255; Matsunaga, T. et al., 1998, *Blood*, 92:452-61). At different time points after transplantation (4 to 8 weeks), mice can be sacrificed to obtain bone marrow cells which then can be cultured in suspension until various assays are performed for X-Gal and cell markers as described earlier. These cells also can be submitted to a secondary colony assay in MC or secondary transplantation into SCID NOD mice. Furthermore, MC colonies derived from these cells can be analyzed for the presence of integrated vector DNA by the method illustrated in Figure 8. The expression and integration data together allow conclusions about the repopulation efficiency and about potential position effects.

B. Optimization of ΔAd.AAV^{fx} vectors for γ r-globin expression in hematopoietic stem cells:

One specific example of the invention is (a) to construct retargeted hybrid vector with the γ -globin as the transgene under the control of erythroid cell specific promoter, (b) to analyze the level and kinetics of γ -globin expression after transduction with hybrid vectors in *in vitro* and *in vivo* assays, (c) if required, to protect gene expression from position effects using γ -globin LCRs or insulators incorporated into hybrid vectors, and (d) to study whether γ -globin introns or heterologous introns can increase γ -globin expression.

Another central issue of the invention is to demonstrate that hybrid vectors can accommodate larger transgenes than rAAV and retroviruses. The insert size limitation of these vectors is 5kb. Transgene cassettes up to 8kb can be inserted into hybrid vectors as described. The maximal insert size may be about 14kb, if hybrid vectors are produced on the basis of E2a and/or E4 deleted rAd vectors in corresponding packaging cell lines. The maximal insert size in hybrid vectors is dictated by the packaging limit of first generation vectors (Ad.AAV) (<36kb) which are necessary intermediates for hybrid virus production at large scale. It is expected that stability and titer of Ad.AAV vectors with an 8kb globin gene cassette is comparable to the vector containing the 2.5-3.5kb cassette used in Ad.AAVBG, Ad.AAV1, and Ad.AAVSNori. The following example experiments address these issues.

Production of ΔAd.AAV^{fx} with large globin expression cassettes:

In order to improve the condition of sickle cell disease, the expression level of the transferred γ-lobin gene must be at least 50% of that of each endogenous βgene. These levels of transgene expression can only be achieved by using optimal expression cassettes, including extended LCRs and intron containing gamma genes. (Forrester, W.C., et al., 1986, *Proc. Natl. Acad. Sci. USA* 83, 1359-1363; Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in*

Hematology, 35, 105-111; Martin, D. et al., 1996, *Current Opinion in Genetics and Development*, 6:488-95), So far, most of the γ-globin expression cassettes are designed for retroviral and rAAV vectors, thus, less than 5kb and have to be devoid of internal splice sites or poly adenylation signals. With integrating vectors described herein, it is possible to go beyond this size limitation. This allows one to improve γ -globin expression in bone marrow cells in terms of an adequate expression level and long term persistence. For this purpose, γ -globin constructs developed by Li et al (Emery, D. W., et al. 1999 *Hum Gene Ther* 10:877-88; Li, Q., et al. 1999. *Blood* 93:2208-16) or by Ellis et al (Ellis, J., et al., 1996, *EMBO J.*, 15, 562-568; Ellis, J., et al., 1997, *Nucleic Acids Res.* 25, 1296-1302) is chosen.

(i) The first cassette contains a γ -globin expression unit used in retroviral vectors. This allows for a direct comparison between the two systems. This construct includes the beta promoter from -127 to the beta initiation codon, which is connected in frame with the gamma coding region. This beta promoter is combined with the 300bp HS40 derived from the human alpha globin locus, which acts as a strong enhancer for globin expression. The globin gene is the 1.1kb version with intron 1 and partially deleted intron 2. A second cassette is generated containing the HS40 beta promoter and gamma globin gene with the complete 3.3kb gamma globin gene.

(ii) The second construct contains the 6.5kb beta μLCR, which confer a dominant chromatin opening activity and an adequate level of gamma globin expression in transgenic mice. The LCR is linked to the short 1.1kb version of the gamma globin gene or the complete 3.3kb gamma gene.

(iii) Additional globin expression cassette can be generated which include insulators, MARs or SARs, as well as other elements that can improve transgene expression from integrated vectors or in transgenic animals, like introns derived from the HPRT or hGH genes (Chung, J.H., et al., 1997, *Proc. Natl. Acad. Sci. USA* 94, 575-580; Dunaway, M., et al, 1993, *Mol. Cell. Biol.*, 17, 182-189; Felsenfeld, G., et al., 1996,

Proc. Natl. Acad. Sci. USA 93, 93840-9388; Klehr, D., et al., 1991, *Biochemistry*, 30, 1264-1270).

Transduction studies with ΔAd.AAVfx-globin vectors:

Transduction studies with globin-hybrid vectors are performed as described earlier (Steinwaerder, D. S., et al. 1999. *J Virol* 73:9303-13). Transduced CD34+ cells are submitted to differentiation in colony assays or analyzed *in vivo* expansion assays in SCID-NOD mice. MC-colonies or bone marrow cells from experimental mice are analyzed for globin expression. Gamma-globin expression is measured using fluorescent anti-gamma-globin antibodies. RNAase protection studies can be performed to specifically quantitate gamma globin mRNA in comparison with -globin RNA. For these studies around 10^4-10^5 cells are needed per test.

Position effects:

In the absence of the LCR, globin genes are subjected to strong position effects when they are transferred into cultured CD34+ cells or erythroleukemic lines (Fraser, P., et al., 1998, *Curr. Opinion in Cell Bio.*, 10, 361-365; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111). Another concern is that site-specific integration of ΔAd.AAV/rep vectors into AAVS1 may silence transgene expression. If silencing happens, it can be overcome by incorporating LCRs such as the 6.5kb □ globin μLCR (Ellis, J., et al., 1996, *EMBO J.*, 15, 562-568; Grosveld, F., et a.l, 1998, *Seminars in Hematology*, 35, 105-111) or insulators into □Ad.AAV based expression units. Insulators are DNA elements that protect an integrated reporter gene from chromosomal position effects or that block enhancer activated transcription from a downward promoter. Insulator elements are known for Drosophila melanogaster genes (Gypsy, suppressor of Hairy wing, scs, scs', Fab-7). for the chicken beta-globin gene (HS4) and for the T cell receptor (BEAD1; 14, 21.25). Specifically, the Drosophila gypsy or the beta globin insulator can be inserted as two copies flanking the globin expression cassette into hybrid vectors. The position effects can be examined in transduced MC-colonies based on the

analysis of integrated vector DNA (see Fig. 29) and gamma-globin mRNA quantification. Analogous studies can be performed on transduced human bone marrow cells obtained after transplantation of infected CD34+ cells into SCID-NOD mice.

Intron effects on gamma-globin expression:

A number of reports reveal that the deletion of globin introns, particularly the second intron of the beta and gamma genes, decrease globin mRNA stability and thus the expression level (Antoniou, M. et al., 1998, *Nucleic Acid Res.*, 26:721-9). RNA viruses such as onco-retro, lenti- and foami viruses are problematic as vehicles for intron-containing transgenes. Because ΔAd.AAV is a DNA virus, it should package globin introns and LCRs if necessary without the decreased titers and rearrangements observed with retroviral vectors.

APPENDIX I

HUMAN AND ANIMAL ADENOVIRUSES AVAILABLE FROM AMERICAN TYPE CULTURE COLLECTION

1: Adenovirus Type 21 ATCC VR-1099 (NIAID V-221-002-014)
2: SA18 (Simian adenovirus 18) ATCC VR-943 Classification
3: SA17 (Simian adenovirus 17) ATCC VR-942 Classification
4: Adenovirus Type 47 ATCC VR-1309 Classification: Adenov
5: Adenovirus Type 44 ATCC VR-1306 Classification: Adenov
6: Avian adenovirus Type 4 ATCC VR-829 Classification: Ad
7: Avian adenovirus Type 5 ATCC VR-830 Classification: Ad
8: Avian adenovirus Type 7 ATCC VR-832 Classification: Ad
9: Avian adenovirus Type 8 ATCC VR-833 Classification: Ad
10: Avian adenovirus Type 9 ATCC VR-834 Classification: Ad
11: Avian adenovirus Type 10 ATCC VR-835 Classification: A
12: Avian adenovirus Type 2 ATCC VR-827 Classification: Ad
13: Adenovirus Type 45 ATCC VR-1307 Classification: Adenov
14: Adenovirus Type 38 ATCC VR-988 Permit: PHS permit requ
15: Adenovirus Type 46 ATCC VR-1308 Classification: Adenov
16: Simian adenovirus ATCC VR-541 Classification: Adenovir
17: SA7 (Simian adenovirus 16) ATCC VR-941 Classification:
18: Frog adenovirus (FAV-1) ATCC VR-896 Classification: Ad
19: Adenovirus Type 48 (candidate) ATCC VR-1406 Classifica
20: Adenovirus Type 42 ATCC VR-1304 Classification: Adenov

21: Adenovirus type 49 (candidate) ATCC VR-1407 Classifica
22: Adenovirus Type 43 ATCC VR-1305 Classification: Adenov
23: Avian adenovirus Type 6 ATCC VR-831 Permit: USDA permi
24: Avian adenovirus Type 3 (Inclusion body hepatitis virus)
25: Bovine adenovirus Type 3 ATCC VR-639 Classification: A
26: Bovine adenovirus Type 6 ATCC VR-642 Permit: USDA perm
27: Canine adenovirus ATCC VR-800 Classification: Adenovir
28: Bovine adenovirus Type 5 ATCC VR-641 Permit: USDA perm
29: Adenovirus Type 36 ATCC VR-913 Classification: Adenovi
30: Ovine adenovirus type 5 ATCC VR-1343 Classification: A
31: Adenovirus Type 29 ATCC VR-272 Classification: Adenovi
32: Swine adenovirus ATCC VR-359 Classification: Adenoviru
33: Bovine adenovirus Type 4 ATCC VR-640 Permit: USDA perm
34: Bovine adenovirus Type 8 ATCC VR-769 Permit: USDA perm
35: Bovine adenovirus Type 7 ATCC VR-768 Permit: USDA perm
36: Adeno-associated virus Type 2 (AAV-2H) ATCC VR-680 Cla
37: Adenovirus Type 4 ATCC VR-4 Classification: Adenovirus
38: Adeno-associated virus Type 3 (AAV-3H) ATCC VR-681 Cla
39: Peromyscus adenovirus ATCC VR-528 Classification: Aden
40: Adenovirus Type 15 ATCC VR-661 Classification: Adenovi
41: Adenovirus Type 20 ATCC VR-662 Classification: Adenovi
42: Chimpanzee adenovirus ATCC VR-593 Classification: Aden
43: Adenovirus Type 31 ATCC VR-357 Classification: Adenovi
44: Adenovirus Type 25 ATCC VR-223 Classification: Adenovi

45: <u>Chimpanzee adenovirus ATCC VR-592 Classification: Aden</u>
46: <u>Chimpanzee adenovirus ATCC VR-591 Classification: Aden</u>
47: <u>Adenovirus Type 26 ATCC VR-224 Classification: Adenovi</u>
48: <u>Adenovirus Type 19 ATCC VR-254 Classification: Adenovi</u>
49: <u>Adenovirus Type 23 ATCC VR-258 Classification: Adenovi</u>
50: <u>Adenovirus Type 28 ATCC VR-226 Classification: Adenovi</u>
51: <u>Adenovirus Type 6 ATCC VR-6 Classification: Adenovirus</u>
52: <u>Adenovirus Type 2 Antiserum: ATCC VR-1079 AS/Rab (NIA</u>
53: <u>Adenovirus Type 6 ATCC VR-1083 (NIAID V-206-003-014)</u>
54: <u>Ovine adenovirus type 6 ATCC VR-1340 Classification: A</u>
55: <u>Adenovirus Type 3 ATCC VR-847 (Derived from NIAID V-20)</u>
56: <u>Adenovirus Type 7 ATCC VR-7 Classification: Adenovirus</u>
57: <u>Adenovirus Type 39 ATCC VR-932 Classification: Adenovi</u>
58: <u>Adenovirus Type 3 ATCC VR-3 Classification: Adenovirus</u>
59: <u>Bovine adenovirus Type 1 ATCC VR-313 Classification: A</u>
60: <u>Adenovirus Type 14 ATCC VR-15 Classification: Adenovir</u>
61: <u>Adenovirus Type 1 ATCC VR-1078 (NIAID V-201-001-014)</u>
62: <u>Adenovirus Type 21 ATCC VR-256 Classification: Adenovi</u>
63: <u>Adenovirus Type 18 ATCC VR-1095 (NIAID V-218-003-014)</u>
64: <u>Baboon adenovirus ATCC VR-275 Classification: Adenovir</u>
65: <u>Adenovirus Type 10 ATCC VR-11 Classification: Adenovir</u>
66: <u>Adenovirus Type 33 ATCC VR-626 Classification: Adenovi</u>

67: <u>Adenovirus Type 34 ATCC VR-716 Classification: Adenovi</u>

68: <u>Adenovirus Type 15 ATCC VR-16 Classification: Adenovir</u>

69: <u>Adenovirus Type 22 ATCC VR-257 Classification: Adenovi</u>

70: <u>Adenovirus Type 24 ATCC VR-259 Classification: Adenovi</u>

71: <u>Adenovirus Type 17 ATCC VR-1094 (NIAID V-217-003-014)</u>

72: <u>Adenovirus Type 4 ATCC VR-1081 (NIAID V-204-003-014)</u>

73: <u>Adenovirus Type 16 ATCC VR-17 Classification: Adenovir</u>

74: <u>Adenovirus Type 17 ATCC VR-18 Classification: Adenovir</u>

75: <u>Adenovirus Type 16 ATCC VR-1093 (NIAID V-216-003-014)</u>

76: <u>Bovine adenovirus Type 2 ATCC VR-314 Classification: A</u>

77: <u>SV-30 ATCC VR-203 Classification: Adenovirus, Simian (</u>

78: <u>Adenovirus Type 32 ATCC VR-625 Classification: Adenovi</u>

79: <u>Adenovirus Type 20 ATCC VR-255 Classification: Adenovi</u>

80: <u>Adenovirus Type 13 ATCC VR-14 Classification: Adenovir</u>

81: <u>Adenovirus Type 14 ATCC VR-1091 (NIAID V-214-001-014)</u>

82: <u>Adenovirus Type 18 ATCC VR-19 Classification: Adenovir</u>

83: <u>SV-39 ATCC VR-353 Classification: Adenovirus, Simian (</u>

84: <u>Adenovirus Type 11 ATCC VR-849 (Derived from NIAID V-2</u>

85: <u>Duck adenovirus (Egg drop syndrome) ATCC VR-921 Permi</u>

86: <u>Adenovirus Type 1 ATCC VR-1 Classification: Adenovirus</u>

87: <u>Chimpanzee adenovirus ATCC VR-594 Classification: Aden</u>

88: <u>Adenovirus Type 15 ATCC VR-1092 (NIAID V-215-003-014)</u>

89: Adenovirus Type 13 ATCC VR-1090 (NIAID V-213-003-014)
90: Adenovirus Type 8 ATCC VR-1368 (Derived from NIAID V-20
91: SV-31 ATCC VR-204 Classification: Adenovirus, Simian (
92: Adenovirus Type 9 ATCC VR-1086 (NIAID V-209-003-014)
93: Mouse adenovirus ATCC VR-550 Classification: Adenoviru
94: Adenovirus Type 9 ATCC VR-10 Classification: Adenoviru
95: Adenovirus Type 41 ATCC VR-930 Classification: Adenovi
96: CI ATCC VR-20 Classification: Adenovirus, Simian (Mast
97: Adenovirus Type 40 ATCC VR-931 Classification: Adenovi
98: Adenovirus Type 37 ATCC VR-929 Classification: Adenovi
99: Marble spleen disease virus (Hemorrhagic enteritis virus
100: Adenovirus Type 35 ATCC VR-718 Classification: Adenovi
101: SV-32 (M3) ATCC VR-205 Classification: Adenovirus, Sim
102: Adenovirus Type 28 ATCC VR-1106 (NIAID V-228-003-014)
103: Adenovirus Type 10 ATCC VR-1087 (NIAID V-210-003-014)
104: Adenovirus Type 20 ATCC VR-1097 (NIAID V-220-003-014)
105: Adenovirus Type 21 ATCC VR-1098 (NIAID V-221-011-014)
106: Adenovirus Type 25 ATCC VR-1103 (NIAID V-225-003-014)
107: Adenovirus Type 26 ATCC VR-1104 (NIAID V-226-003-014)
108: Adenovirus Type 31 ATCC VR-1109 (NIAID V-231-001-014)
109: Adenovirus Type 19 ATCC VR-1096 (NIAID V-219-002-014)
110: SV-36 ATCC VR-208 Classification: Adenovirus, Simian (

111: SV-38 ATCC VR-355 Classification: Adenovirus, Simian (

112: SV-25 (M8) ATCC VR-201 Classification: Adenovirus, Sim

113: SV-15 (M4) ATCC VR-197 Classification: Adenovirus, Sim

114: Adenovirus Type 22 ATCC VR-1100 (NIAID V-222-003-014)

115: SV-23 (M2) ATCC VR-200 Classification: Adenovirus, Sim

116: Adenovirus Type 11 ATCC VR-12 Classification: Adenovir

117: Adenovirus Type 24 ATCC VR-1102 (NIAID V-224-003-014)

118: Avian adenovirus Type 1 (Chicken Embryo Lethal Orphan: C

119: SV-11 (M5) ATCC VR-196 Classification: Adenovirus, Sim

120: Adenovirus Type 5 ATCC VR-5 Classification: Adenovirus

121: Adenovirus Type 23 ATCC VR-1101 (NIAID V-223-003-014)

122: SV-27 (M9) ATCC VR-202 Classification: Adenovirus, Sim

123: Avian adenovirus Type 2 (GAL) ATCC V-280 Classificati

124: SV-1 (M1) ATCC VR-195 Classification: Adenovirus, Simi

125: SV-17 (M6) ATCC VR-198 Classification: Adenovirus, Sim

126: Adenovirus Type 29 ATCC VR-1107 (NIAID V-229-003-014)

127: Adenovirus Type 2 ATCC VR-846 Classification: Adenovir

128: SV-34 ATCC VR-207 Classification: Adenovirus, Simian (

129: SV-20 (M7) ATCC VR-199 Classification: Adenovirus, Sim

130: SV-37 ATCC VR-209 Classification: Adenovirus, Simian (

131: SV-33 (M10) ATCC VR-206 Classification: Adenovirus, Si

132: Avian adeno-associated virus ATCC VR-865 Classificatio

133: Adeno-associated (satellite) virus Type 4 ATCC VR-646

134: Adenovirus Type 30 ATCC VR-273 Classification: Adenovi

135: Adeno-associated (satellite) virus Type 1 ATCC VR-645

136: Infectious canine hepatitis (Rubarth's disease, Fox ence

137: Adenovirus Type 27 ATCC VR-1105 (NIAID V-227-003-014)

138: Adenovirus Type 12 ATCC VR-863 (Derived from NIAID V-2

139: Adeno-associated virus Type 2 (molecularly cloned) ATCC

140: Adenovirus Type 7a ATCC VR-848 (Derived from NIAID V-2

What is claimed:

1. A first generation recombinant adenovirus vector a portion of which integrates into a host cell genome, comprising:
   a) A left adenovirus inverted terminal repeat sequence;
   b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
   c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
   d) At least one adenoviral sequence which directs adenoviral replication; and
   e) A right adenoviral inverted terminal repeat sequence,

   wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

2. The adenovirus vector of claim 1, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype.

3. The adenovirus vector of claim 1, wherein the sequence which directs adenoviral replication comprises a sequence on the anti-parallel strand which encodes an adenoviral fiber protein including a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

4. The adenovirus vector of claim 3, wherein the sequence on the anti-sense strand which encodes the fiber tail is from the same serotype as the adenoviral inverted repeat sequence.

5. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:
   a) A left cassette inverted terminal repeat sequence;
   b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the polyadenylation sequence;

d) A promoter sequence 3' to the polyadenylation sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

6. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;

b) A promoter sequence 3' to the left inverted terminal repeat sequence;

c) A transgene sequence 3' to the promoter sequence;

d) A polyadenylation sequence 3' to the transgene sequence; and

e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

7. The adenovirus vector of claim 5 or 6, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

8. The adenovirus vector of claim 5 or 6, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

9. The adenovirus vector of claim 8, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

10. The adenovirus vector of claim 8, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

11. The adenovirus vector of claim 8, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

12. The transgene cassette of claim 5 or 6 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

13. The transgene cassette of claim 5 or 6, further comprising an insulator element.

14. The transgene cassette of claim 5 or 6, further comprising a bacterial origin of replication.

15. The adenoviral vector of claim 1, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

16. A first generation recombinant adenovirus vector which targets a host cell of interest and a portion of which integrates into the host cell genome so targeted, comprising two DNA strands, each strand being antiparallel to the other, the first strand comprising:

a) A left adenovirus inverted terminal repeat sequence;
b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
c) A transgene cassette sequence 3' to the adenoviral packaging sequence;
d) At least one adenoviral sequence which directs adenoviral replication; and
e) A right adenoviral inverted terminal repeat sequence,

wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome, and wherein the second strand comprises a sequence which encodes an adenoviral fiber protein that permits targeting of the vector into the host cell of interest.

17. The adenovirus vector of claim 16, wherein the adenoviral protein includes a fiber tail, a fiber shaft, and a fiber knob, wherein the fiber knob includes a G-H loop region.

18. The adenovirus vector of claim 16, wherein the left and right adenovirus inverted terminal repeat sequences and the packaging sequence are from the same adenoviral serotype.

19. The adenovirus vector of claim 17, wherein the fiber tail is from the same serotype as the left and right adenoviral inverted repeat sequences.

20. The adenovirus vector of claim 17, wherein the fiber shaft is from a different serotype as the left and right adenoviral inverted repeat sequences.

21. The adenovirus vector of claim 20, wherein the fiber shaft is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

22. The adenovirus vector of claim 17, wherein the fiber shaft comprises a shortened length.

23. The adenovirus vector of claim 17, wherein the fiber knob is from a different serotype as the left and right adenoviral inverted repeat sequences.

24. The adenovirus vector of claim 23, wherein the fiber knob is from a serotype selected from a group consisting of serotype 3, 7, 9, 11, and 35.

25. The adenovirus vector of claim 17, wherein the fiber knob is a modified fiber knob protein comprising the G-H loop replaced with a heterologous peptide ligand sequence which binds to at least one surface protein on the host cell of interest.

26. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:
    a) A left cassette inverted terminal repeat sequence;
    b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;
    c) A transgene sequence 3' to the polyadenylation sequence;
    d) A promoter sequence 3' to the polyadenylation sequence; and
    e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

27. The adenovirus vector of claim 16, wherein the transgene cassette sequence comprises:
    a) A left cassette inverted terminal repeat sequence;
    b) A promoter sequence 3' to the left inverted terminal repeat sequence;
    c) A transgene sequence 3' to the promoter sequence;
    d) A polyadenylation sequence 3' to the transgene sequence; and
    e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

28. The adenovirus vector of claim 26 or 27, wherein the left and the right cassette inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

29. The adenovirus vector of claim 26 or 27, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

30. The adenovirus vector of claim 24, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

31. The adenovirus vector of claim 24, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentamycin.

32. The adenovirus vector of claim 24, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

33. The transgene cassette of claim 26 or 27 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

34. The transgene cassette of claim 26 or 27, further comprising an insulator element.

35. The transgene cassette of claim 26 or 27, further comprising a bacterial origin of replication.

36. The adenoviral vector of claim 16, wherein the adenoviral sequences which direct adenoviral replication are selected from a group consisting of E2 and E4; E1, E2 and E4; E2 and E4; and E2, E3, and E4.

37. A recombinant gutless adenovirus vector a portion of which integrates into a host cell genome, comprising:

a) A left adenovirus inverted terminal repeat sequence;
b) An adenoviral packaging sequence 3' to the left adenovirus inverted terminal repeat sequence;
c) A transgene cassette sequence 3' to the adenoviral packaging sequence; and
d) A right adenoviral inverted terminal repeat sequence,

wherein the left and right terminal repeat sequences permit integration of the transgene cassette sequence into the host cell genome.

38. The adenovirus vector of claim 36, wherein the left and right adenovirus inverted repeat sequence and the packaging sequence are from the same adenoviral serotype. *(this is the base vector)*

39. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:

a) A left cassette inverted terminal repeat sequence;
b) A polyadenylation sequence 3' to the left inverted terminal repeat sequence;
c) A transgene sequence 3' to the polyadenylation sequence;
d) A promoter sequence 3' to the polyadenylation sequence; and
e) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

40. The adenovirus vector of claim 36, wherein the transgene cassette sequence comprises:

b) A left cassette inverted terminal repeat sequence;
c) A promoter sequence 3' to the left inverted terminal repeat sequence;
d) A transgene sequence 3' to the promoter sequence;
e) A polyadenylation sequence 3' to the transgene sequence; and
f) A right cassette inverted terminal repeat sequence 3' to the polyadenylation sequence.

41. The adenovirus vector of claim 38 or 39, wherein the left and the right inverted terminal repeat sequences each comprise an adenoviral-associated inverted terminal repeat sequence.

42. The adenovirus vector of claim 38 or 39, wherein the transgene sequence is selected from a group consisting of a therapeutic gene, a selectable gene, and a reporter gene.

43. The adenovirus vector of claim 41, wherein the therapeutic gene is selected from a group consisting of gamma globin, and human alpha-1 anti-trypsin.

44. The adenovirus vector of claim 41, wherein the selectable gene is selected from a group consisting of neomycin, ampicillin, penicillin, tetracyline, and gentomycin.

45. The adenovirus vector of claim 41, wherein the reporter gene is selected from a group consisting of green fluorescent protein, beta galactosidase, alkaline phosphatase.

46. The adenovirus vector of claim 38 or 39 further comprising an inverted repeat sequence located 3' to the left inverted terminal repeat sequence or located 5' to the right inverted terminal repeat sequence.

47. The adenovirus vector of claim 38 or 39, further comprising an insulator element.

48. The adenovirus vector of claim 38 or 39, further comprising a bacterial origin of replication.

49. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 5' portion of a gene of interest.

50. The adenovirus vector of claim 1, wherein the transgene cassette sequence comprises a 3' portion of a gene of interest.

51. A method of producing a resolved gutless adenovirus vector in a suitable cell, said method comprising introducing a first and a second adenovirus vectors of claim 1 or 16 into the cell under suitable conditions so that the recombinant adenovirus vectors undergo homologous recombination thereby producing a resolved gutless adenovirus vector.

52. A resolved gutless adenovirus vector produced by the method of claim 50.

53. The method of claim 50, wherein the first adenovirus vector comprises a transgene cassette having a 5' portion of a gene of interest, and wherein the second adenovirus vector comprises a transgene cassette having a 3' portion of the gene of interest, and wherein a part of the 5' portion overlaps with a part of the 3' portion so that homologous recombination occurs.

54. A method of producing a resolved gutless recombinant Ad vector by homologous recombination in a suitable cell, said method comprising contacting two parental recombinant Ad vectors, each comprising a transgene cassette containing a portion of a selected transgene with a region of overlapping homology, so that the first and second parental recombinant Ad vectors undergo homologous recombination at the region of overlapping homology, resulting in a resolved recombinant gutless Ad vector having both portions of the selected transgene, and wherein the selected transgene is within a transgene cassette flanked by a pair of ITRs.

55. A resolved gutless adenovirus vector produced by the method of claim 53.

56. An adenovirus library comprising a plurality of adenovirus vectors expressing fiber proteins which are displayed and modified by random peptide insertions.

57. The library of claim 55, wherein said fiber protein so displayed comprises a random peptide substituted in the G-H loop of the fiber protein knob domain.

58. A screening method for targeting adenovirus vectors for gene therapy comprising contacting the adenovirus library of claim 55 with a plurality of cells so that the cells are transduced with the adenovirus vectors of the adenovirus library transduction occurs and detecting the cells so transduced.

59. The adenovirus vector of claim 5, 6, 26, 27, 38, or 39 further comprising a nucleotide sequence encoding a rep78 protein.

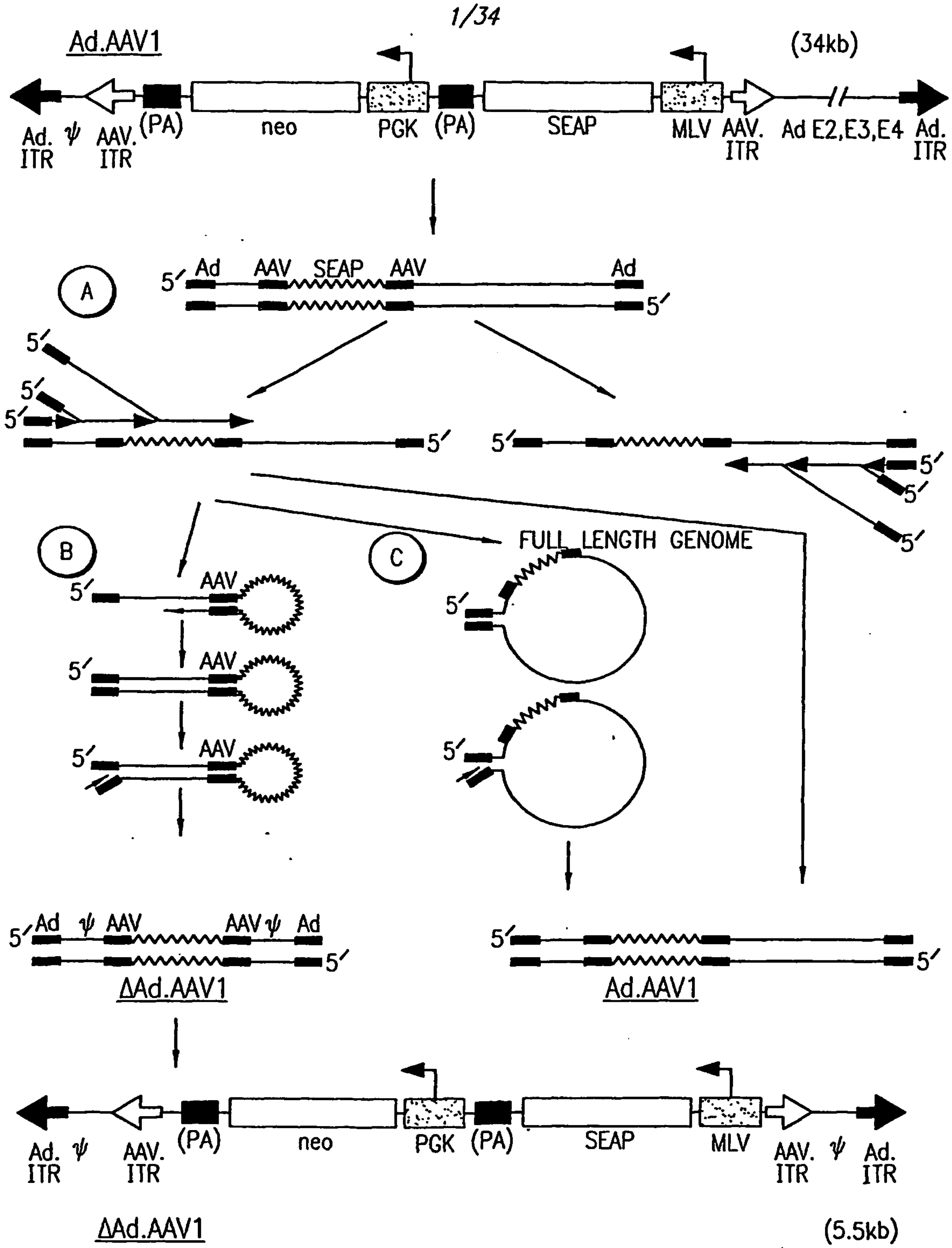

Ad.AAV1
(34kb)
Ad. ITR
ψ
AAV. ITR
(PA)
neo
PGK
(PA)
SEAP
MLV
AAV. ITR
Ad E2,E3,E4
Ad. ITR
A
5′ Ad AAV SEAP AAV Ad
5′
B
C
FULL LENGTH GENOME
AAV
5′
Ad ψ AAV AAV ψ Ad
ΔAd.AAV1
Ad.AAV1
ΔAd.AAV1
(5.5kb)

FIG. 1

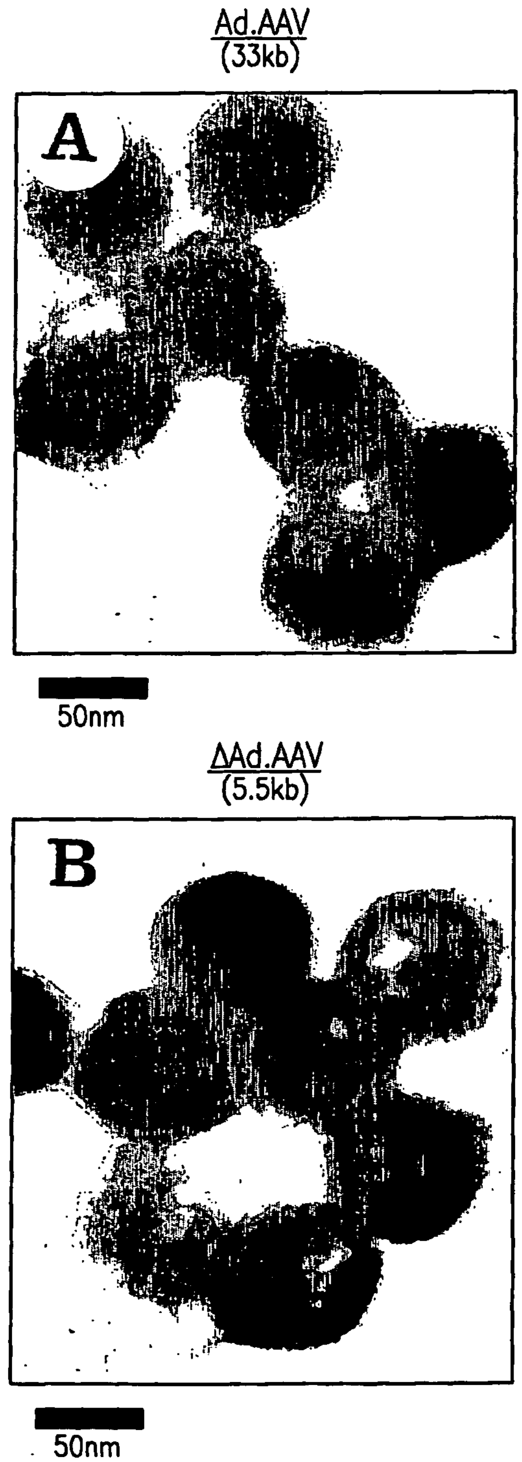
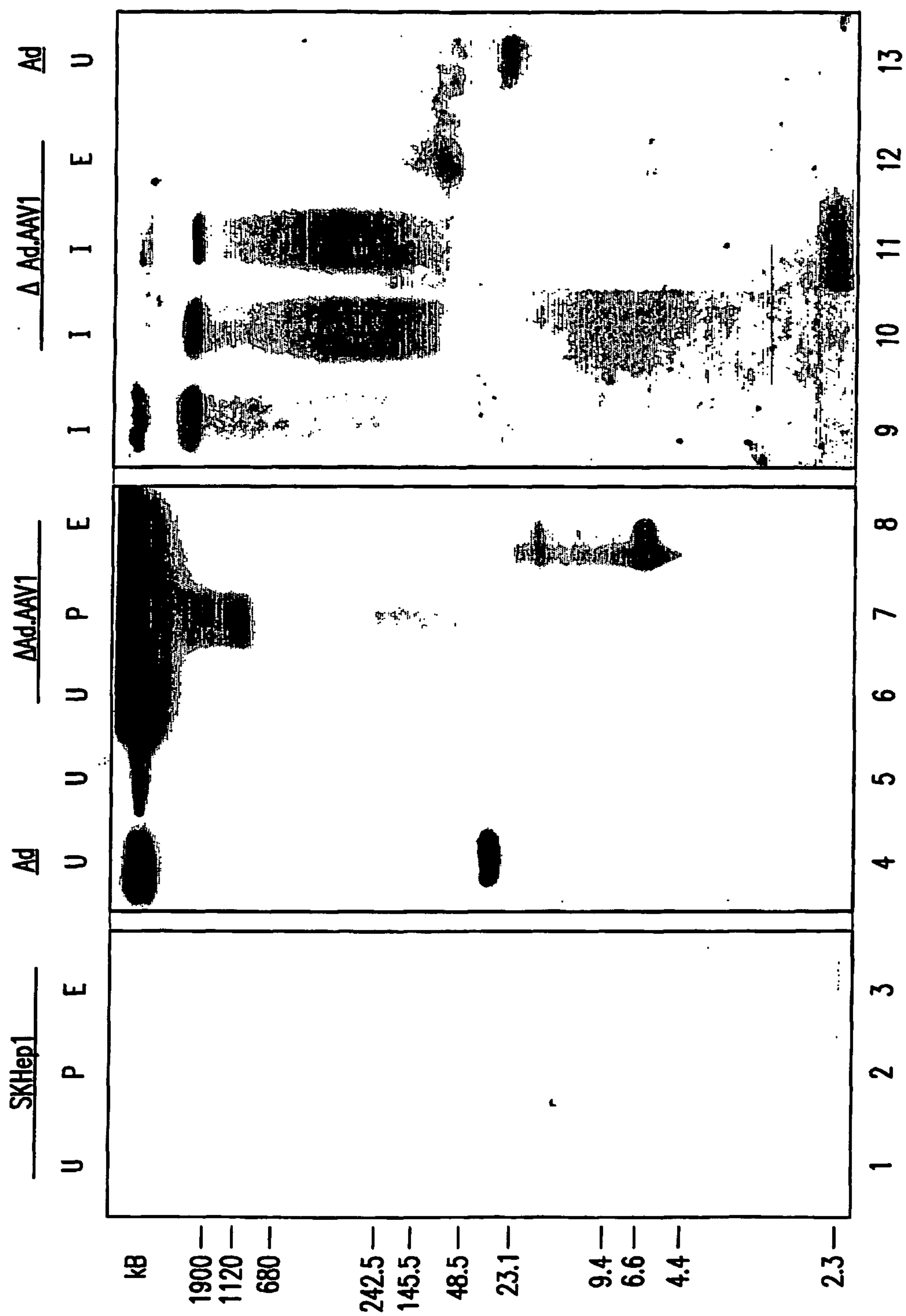


FIG. 2



SKHep1
Ad
ΔAd.AAV1
Δ Ad.AAV1
Ad
U P E U U U P E I I I E U
1 2 3 4 5 6 7 8 9 10 11 12 13
kB
1900
1120
680
242.5
145.5
48.5
23.1
9.4
6.6
4.4
2.3

FIG. 3

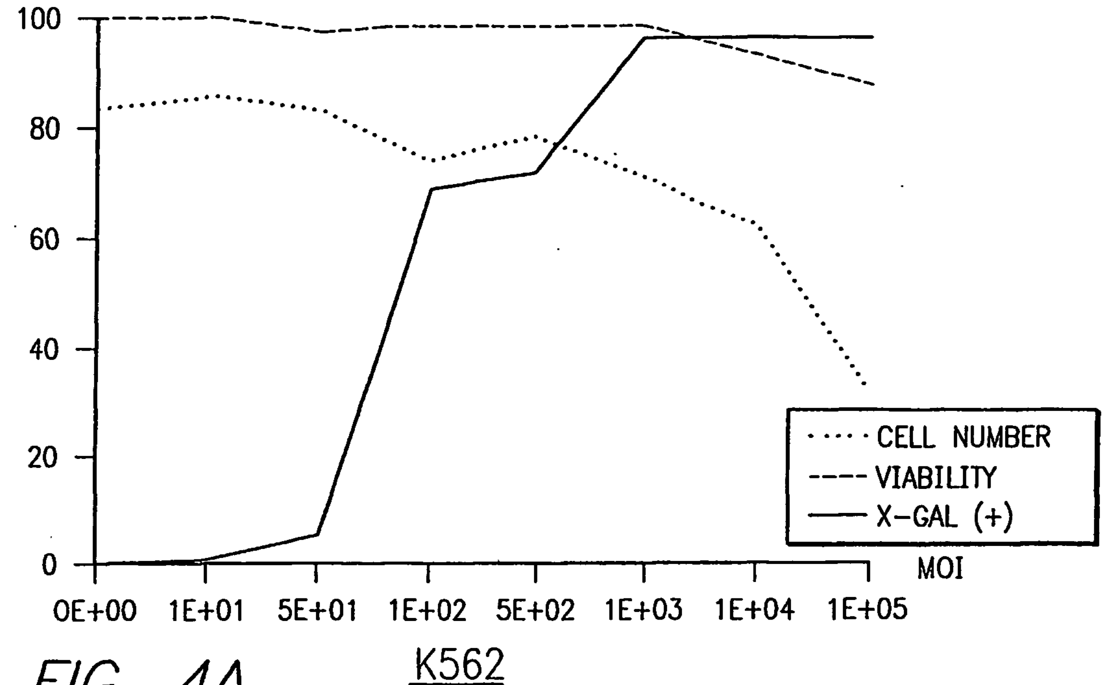

100
80
60
40
20
0
0E+00
1E+01
5E+01
1E+02
5E+02
1E+03
1E+04
1E+05
MOI
CELL NUMBER
VIABILITY
X-GAL (+)
K562

FIG. 4A

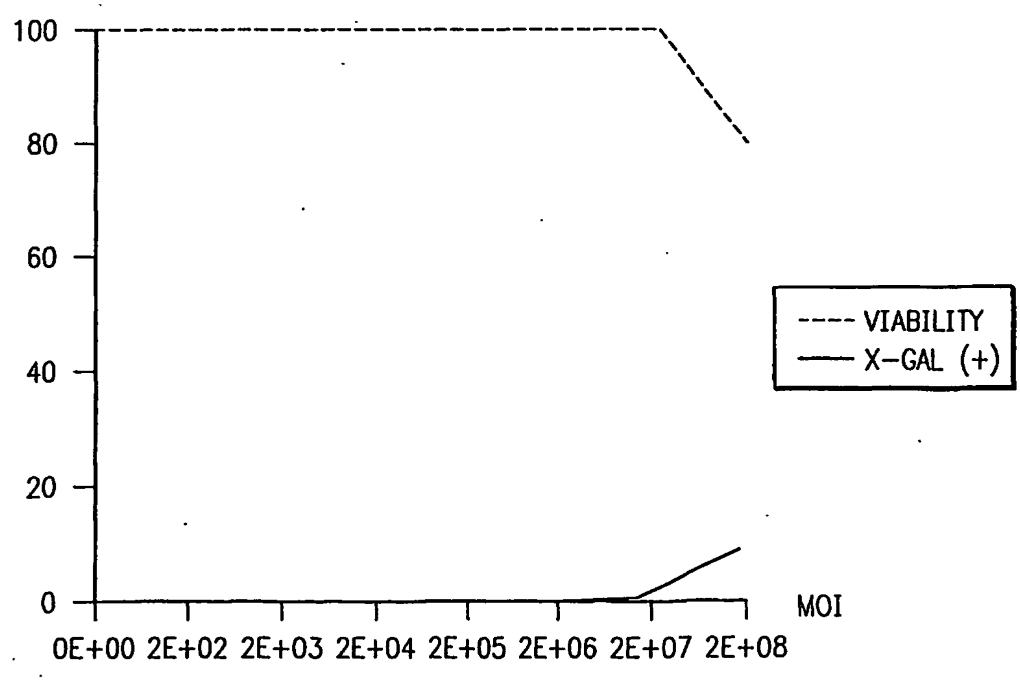

100
80
60
40
20
0
0E+00
2E+02
2E+03
2E+04
2E+05
2E+06
2E+07
2E+08
MOI
VIABILITY
X-GAL (+)
CD34+

FIG. 4B

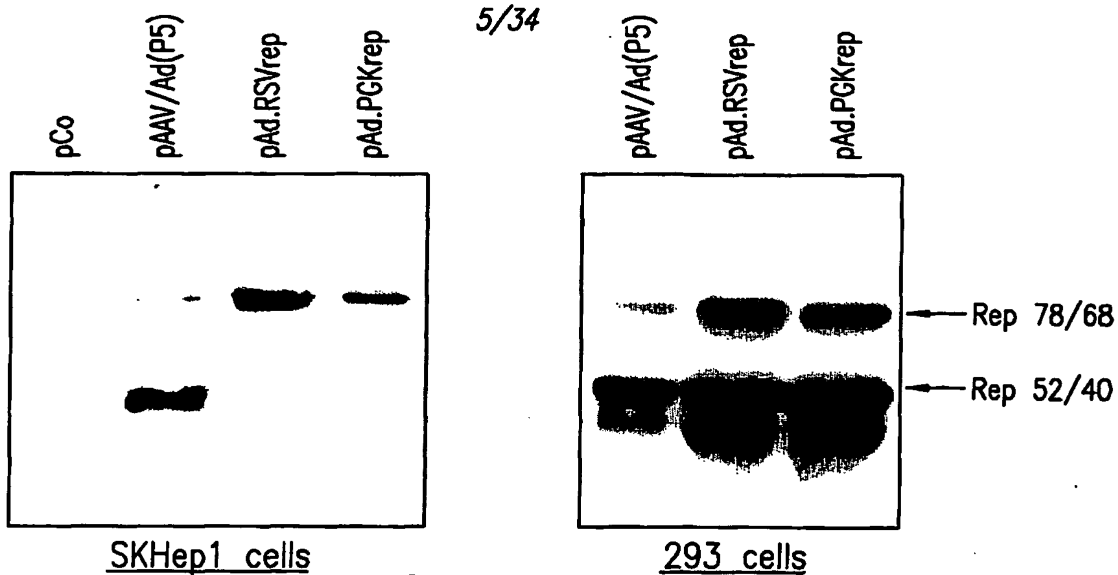

pCo
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
pAAV/Ad(P5)
pAd.RSVrep
pAd.PGKrep
Rep 78/68
Rep 52/40
SKHep1 cells
293 cells

FIG. 5

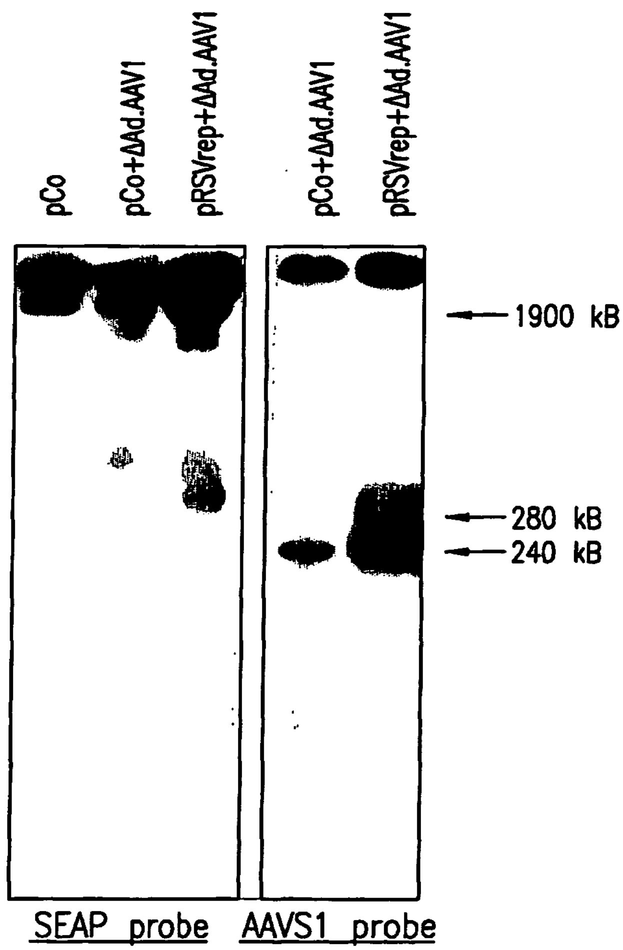

pCo
pCo+ΔAd.AAV1
pRSVrep+ΔAd.AAV1
pCo+ΔAd.AAV1
pRSVrep+ΔAd.AAV1
1900 kB
280 kB
240 kB
SEAP probe
AAVS1 probe

FIG. 6

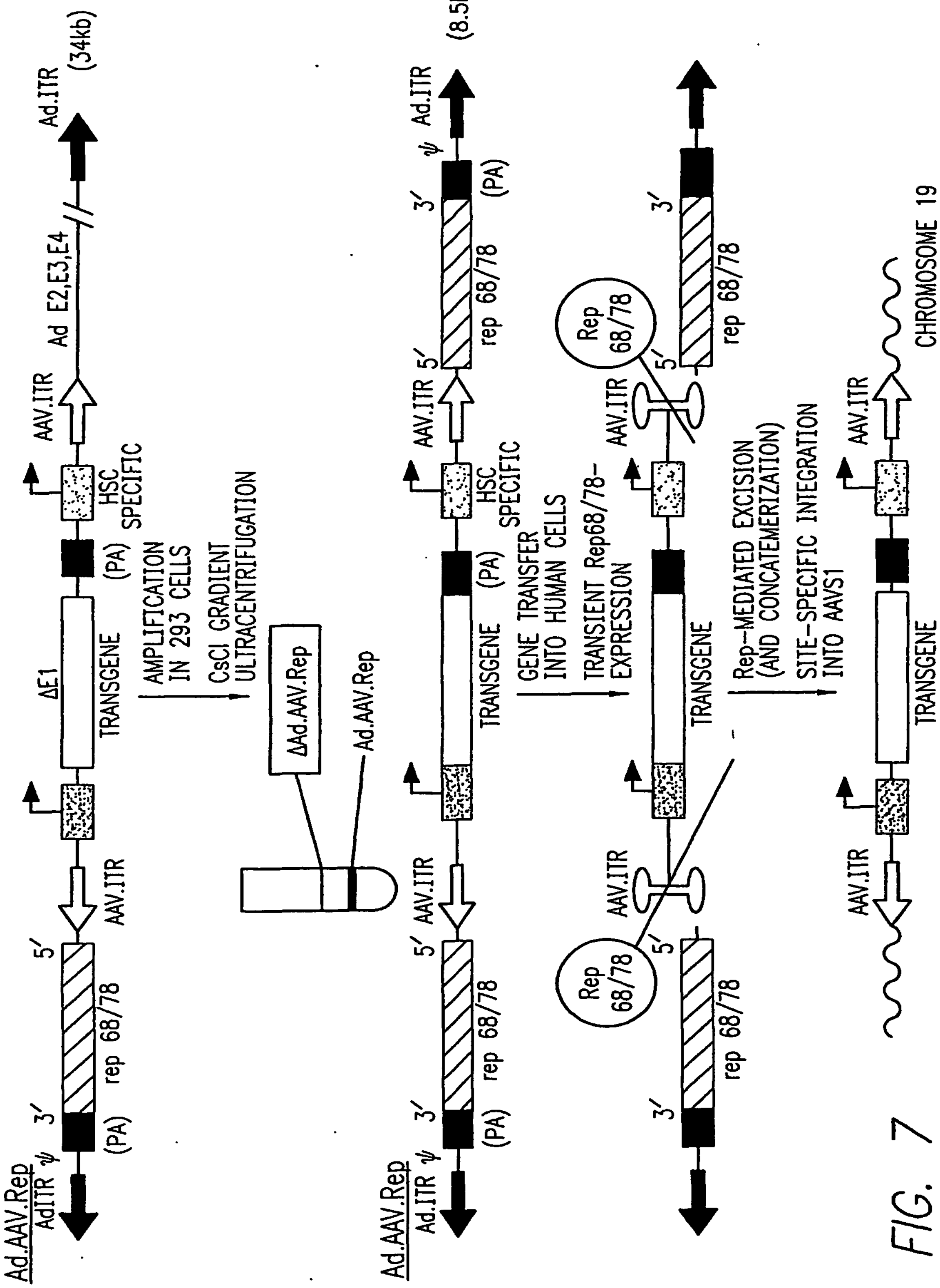

Ad.AAV.Rep
AdITR ψ
(PA)
3′
rep 68/78
5′
AAV.ITR
ΔE1
TRANSGENE
(PA)
HSC SPECIFIC
AAV.ITR
Ad E2,E3,E4
Ad.ITR
(34kb)
AMPLIFICATION IN 293 CELLS
CsCl GRADIENT ULTRACENTRIFUGATION
ΔAd.AAV.Rep
Ad.AAV.Rep
Ad.AAV.Rep
Ad.ITR ψ
(PA)
3′
rep 68/78
5′ AAV.ITR
TRANSGENE
(PA)
HSC SPECIFIC
AAV.ITR
5′
rep 68/78
3′
(PA)
ψ Ad.ITR
(8.5kb)
GENE TRANSFER INTO HUMAN CELLS
TRANSIENT Rep68/78- EXPRESSION
Rep 68/78
3′
rep 68/78
5′
AAV.ITR
TRANSGENE
AAV.ITR
Rep 68/78
5′
rep 68/78
3′
Rep-MEDIATED EXCISION (AND CONCATEMERIZATION)
SITE-SPECIFIC INTEGRATION INTO AAVS1
AAV.ITR
TRANSGENE
AAV.ITR
CHROMOSOME 19

FIG. 7

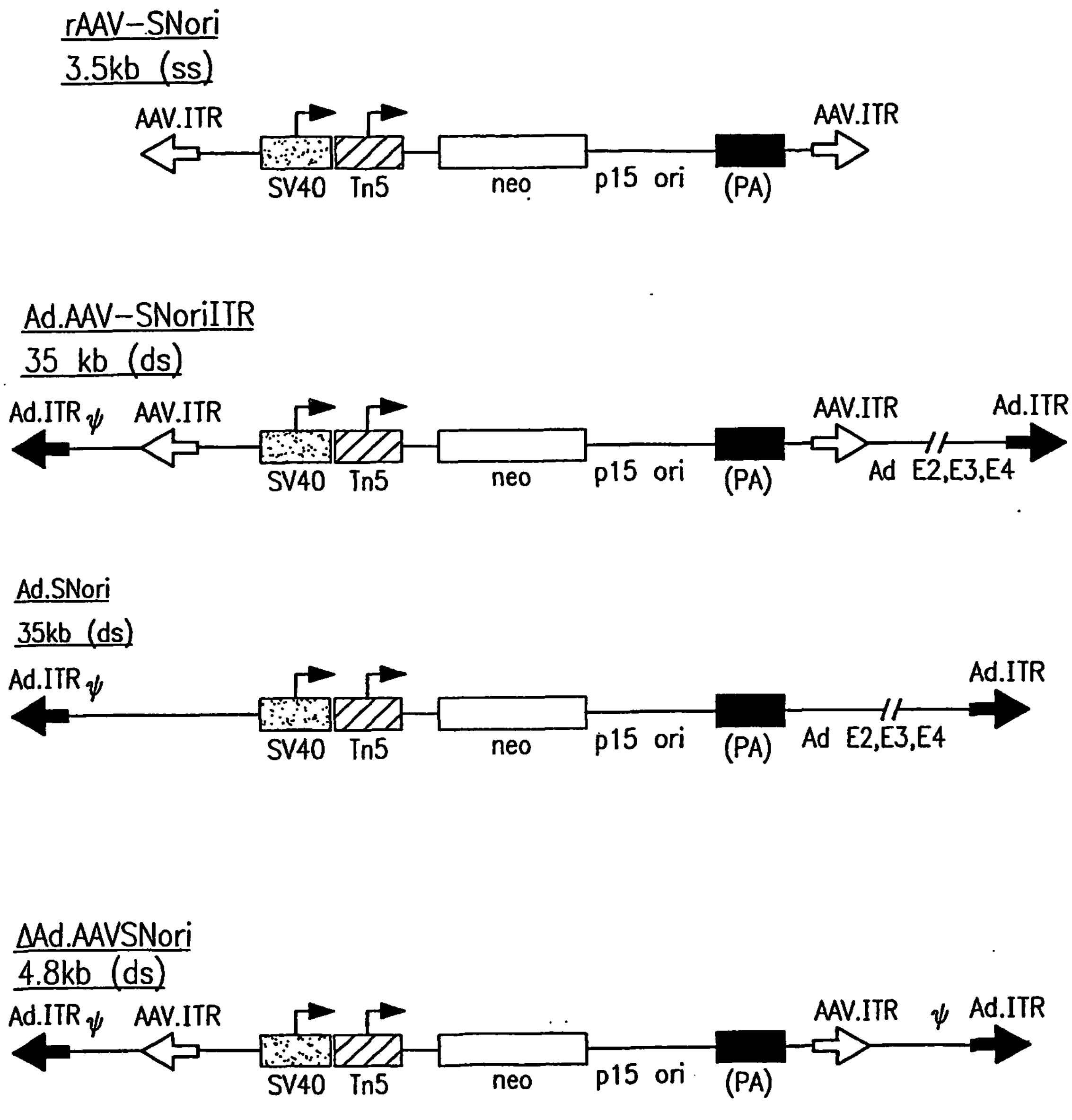

rAAV-SNori
3.5kb (ss)
AAV.ITR
AAV.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad.AAV-SNoriITR
35 kb (ds)
Ad.ITR ψ
AAV.ITR
AAV.ITR
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad E2,E3,E4
Ad.SNori
35kb (ds)
Ad.ITR ψ
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)
Ad E2,E3,E4
ΔAd.AAVSNori
4.8kb (ds)
Ad.ITR ψ
AAV.ITR
AAV.ITR
ψ
Ad.ITR
SV40
Tn5
neo
p15 ori
(PA)

FIG. 8A

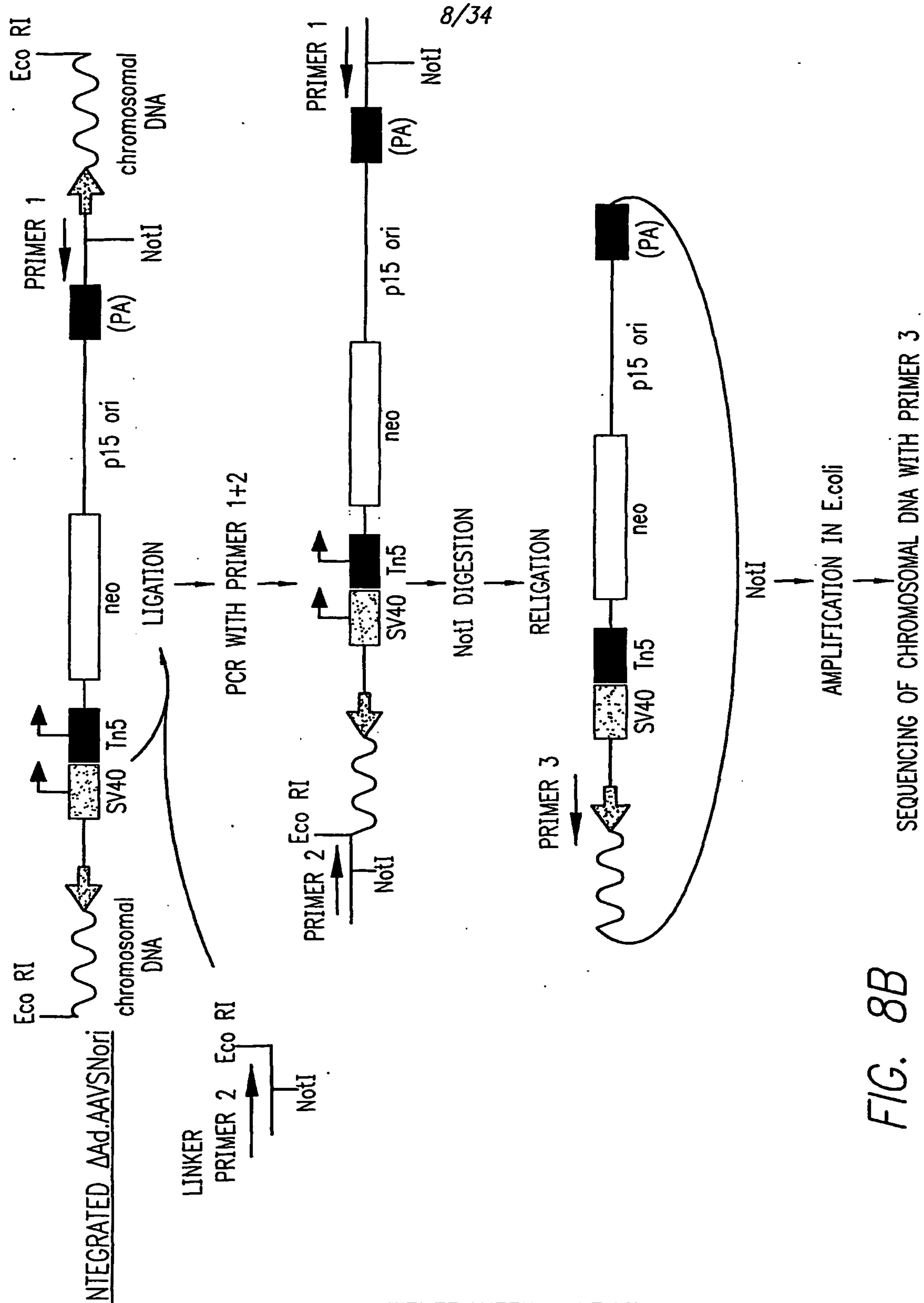

INTEGRATED ΔAd.AAVSNori
Eco RI
chromosomal DNA
SV40
Tn5
neo
p15 ori
(PA)
PRIMER 1
NotI
LINKER
PRIMER 2
LIGATION
PCR WITH PRIMER 1+2
NotI DIGESTION
RELIGATION
PRIMER 3
AMPLIFICATION IN E.coli
SEQUENCING OF CHROMOSOMAL DNA WITH PRIMER 3

FIG. 8B

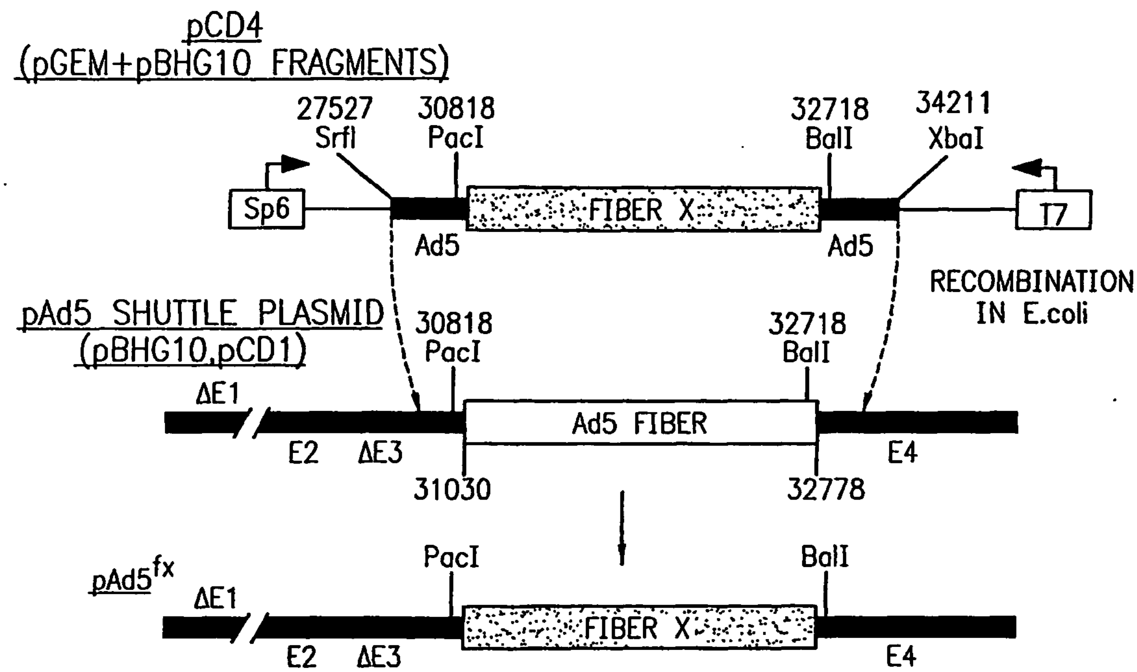

pCD4
(pGEM+pBHG10 FRAGMENTS)
27527
SrfI
30818
PacI
32718
BalI
34211
XbaI
Sp6
FIBER X
T7
Ad5
Ad5
RECOMBINATION
IN E.coli
pAd5 SHUTTLE PLASMID
(pBHG10,pCD1)
30818
PacI
32718
BalI
ΔE1
Ad5 FIBER
E2
ΔE3
E4
31030
32778
pAd5fx
PacI
BalI
ΔE1
FIBER X
E2
ΔE3
E4

FIG. 9

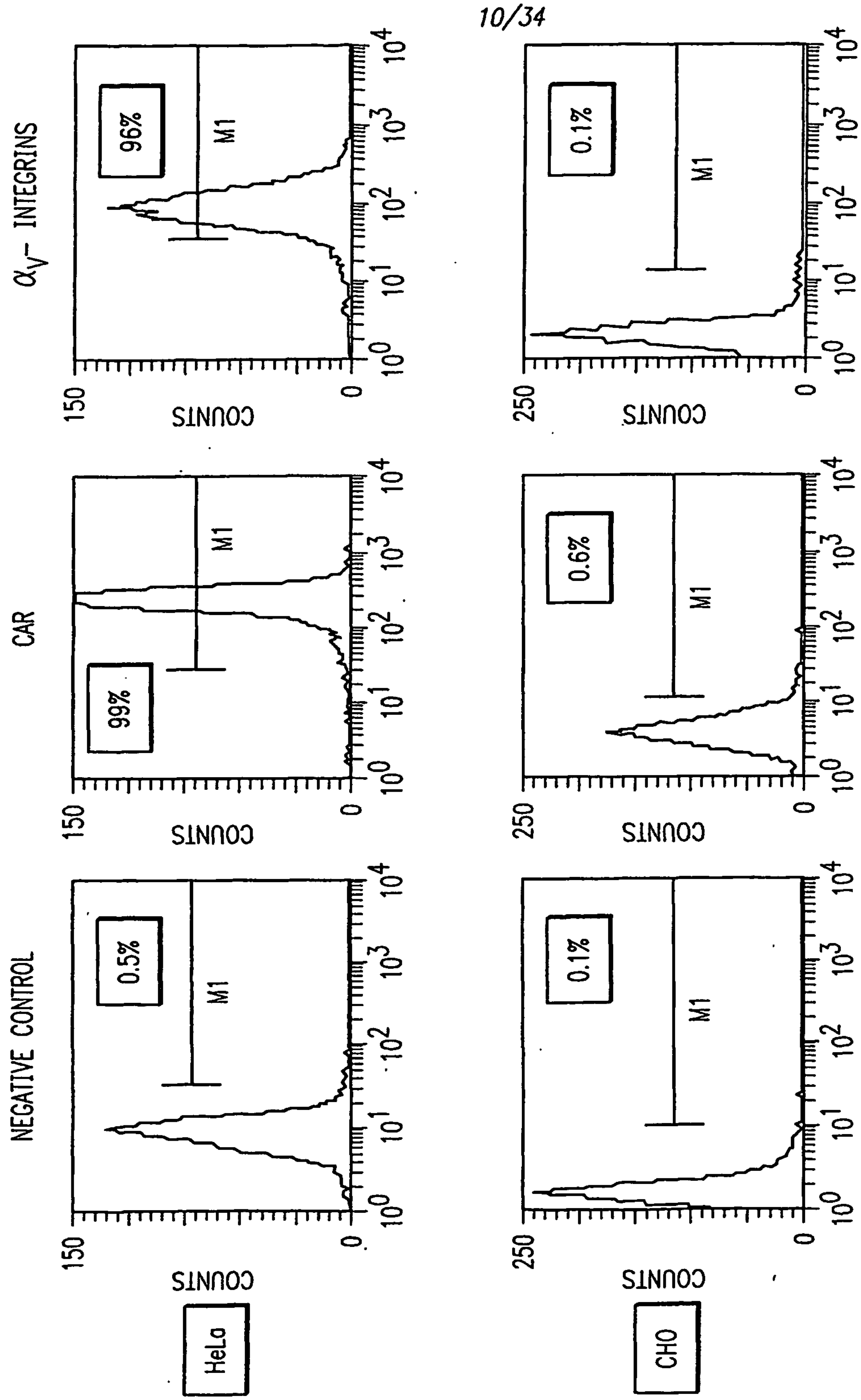

NEGATIVE CONTROL
CAR
α_V– INTEGRINS
HeLa
CHO
COUNTS
M1
0.5%
99%
96%
0.1%
0.6%
0.1%
150
250
0
10^0
10^1
10^2
10^3
10^4

FIG. 10–i

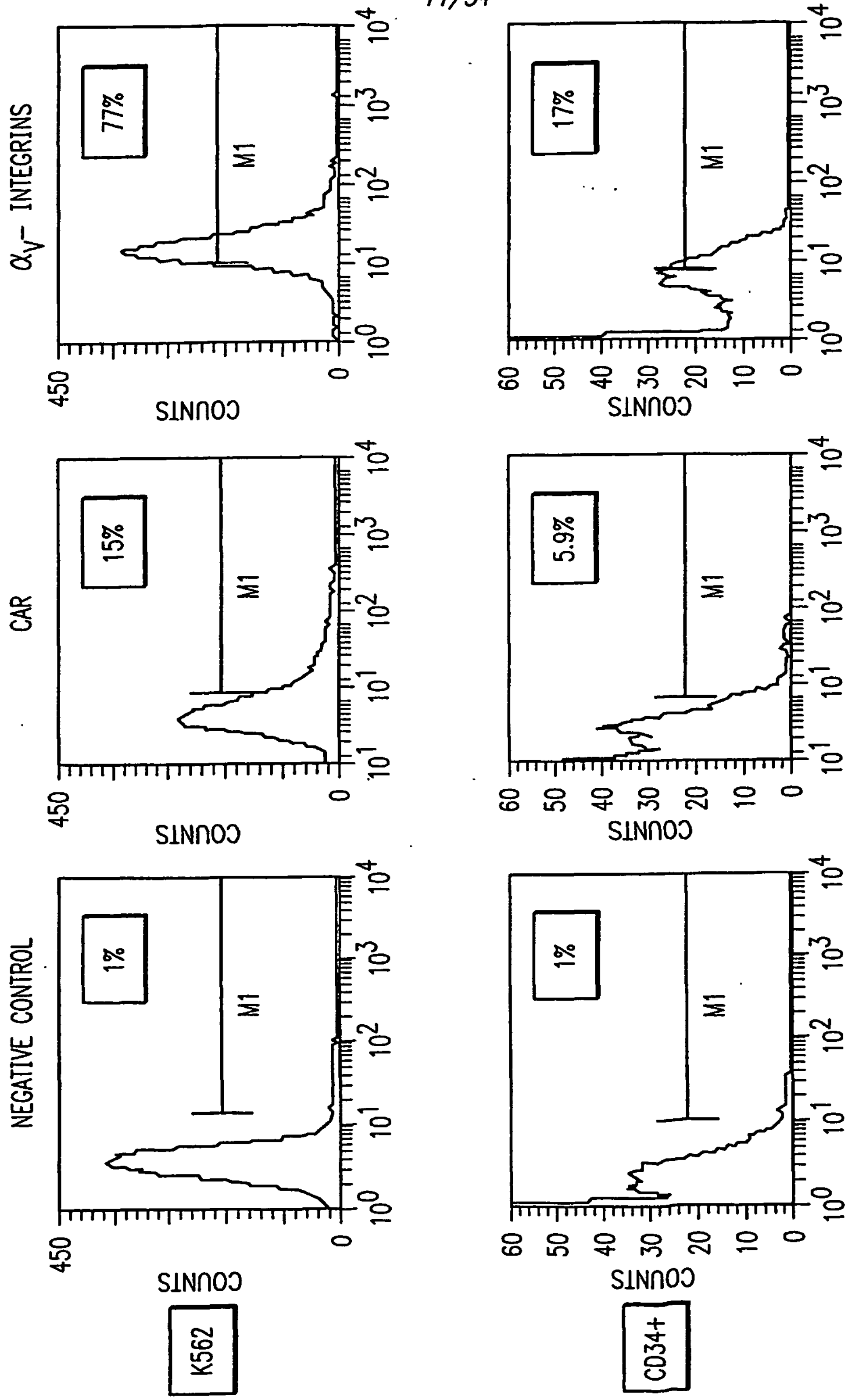
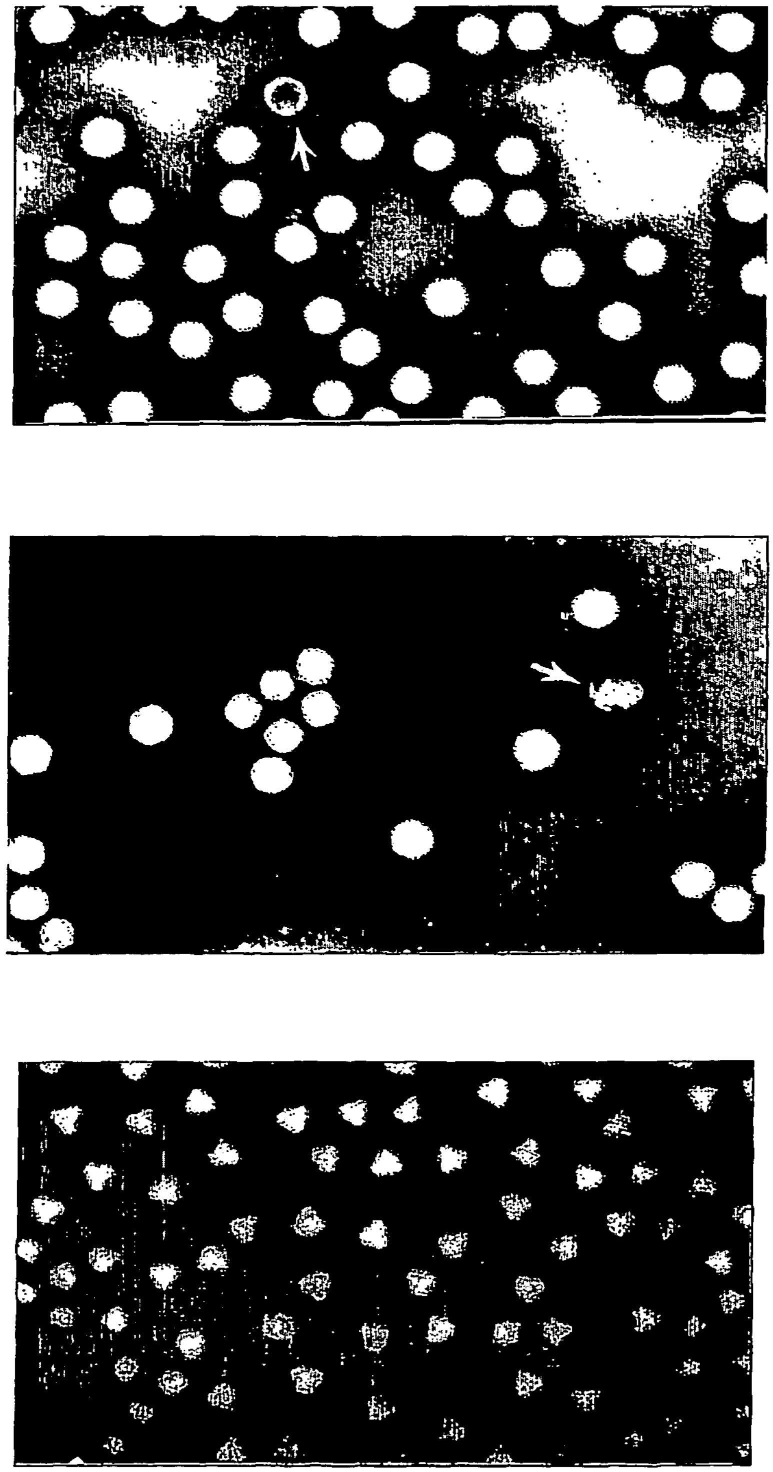

K562
CD34+
NEGATIVE CONTROL
CAR
α_V- INTEGRINS
COUNTS
M1
1%
15%
77%
1%
5.9%
17%

FIG. 10—ii



Ad 35

Ad 9

Ad 5

FIG. 11

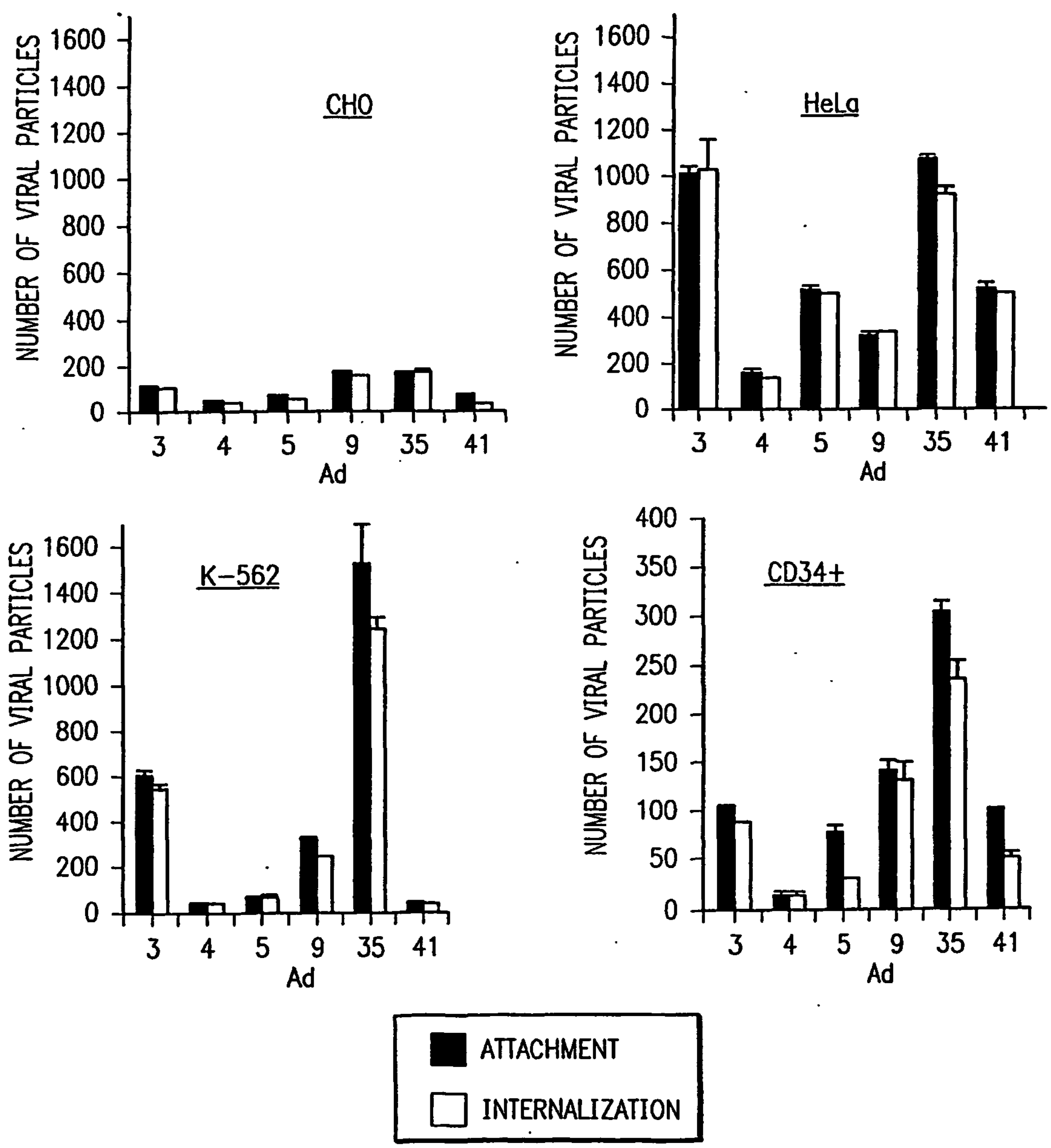

CHO
HeLa
K-562
CD34+
NUMBER OF VIRAL PARTICLES
1600
1400
1200
1000
800
600
400
200
0
400
350
300
250
200
150
100
50
3
4
5
9
35
41
Ad
ATTACHMENT
INTERNALIZATION

FIG. 12

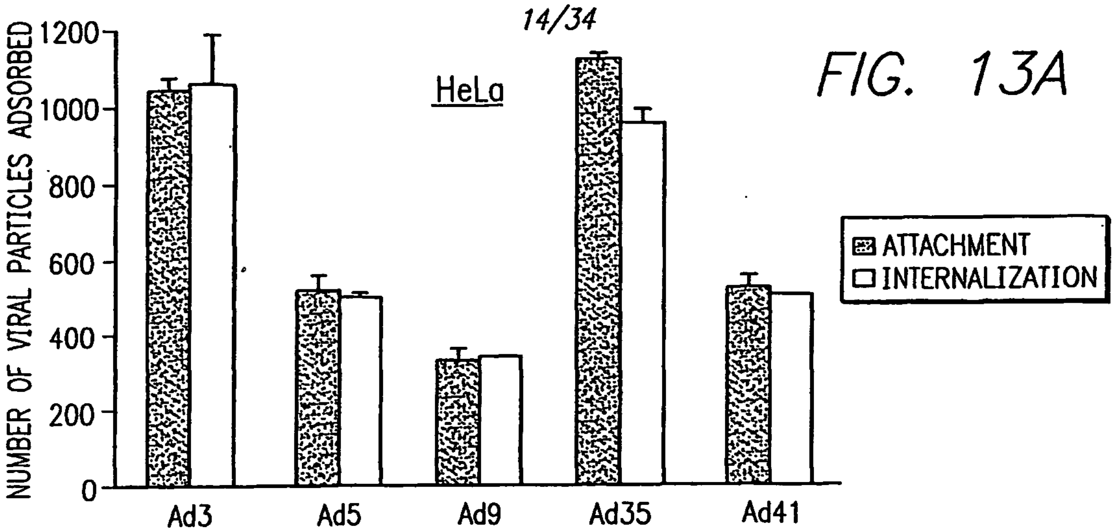


FIG. 13A

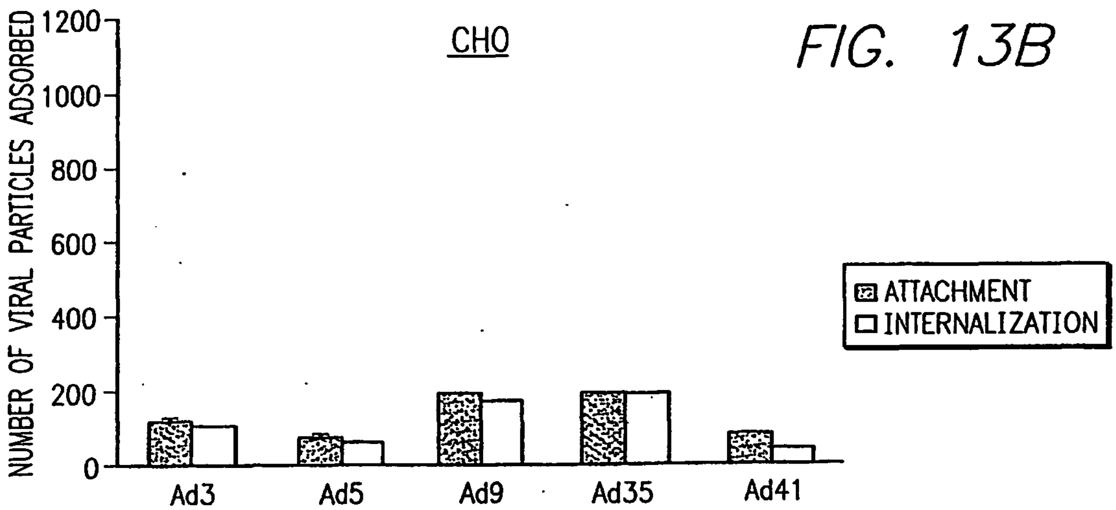


FIG. 13B

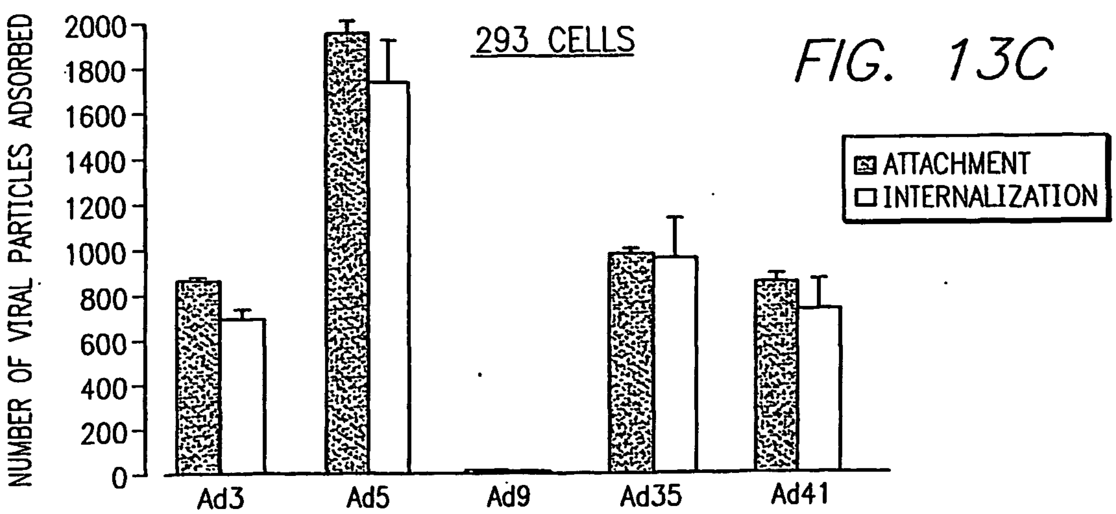


FIG. 13C

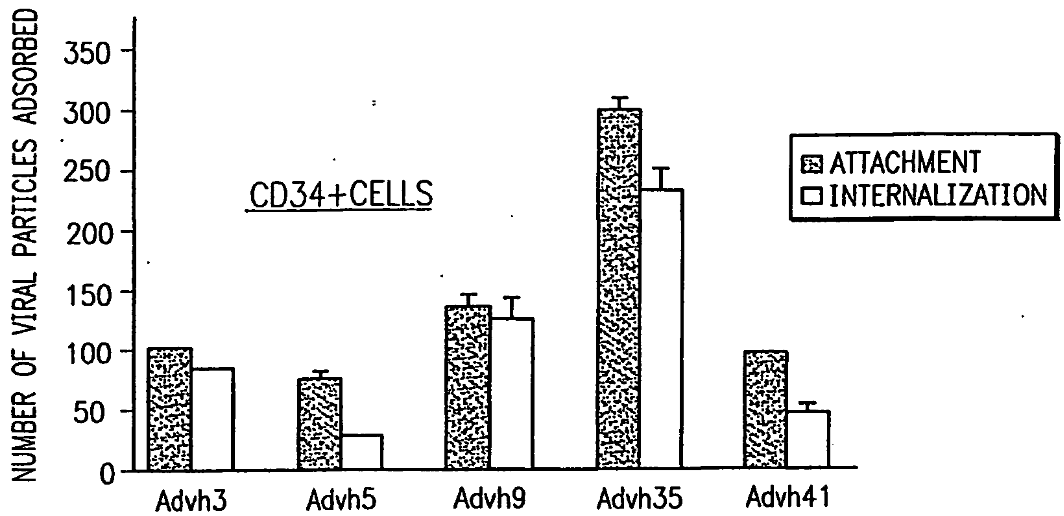


FIG. 14A

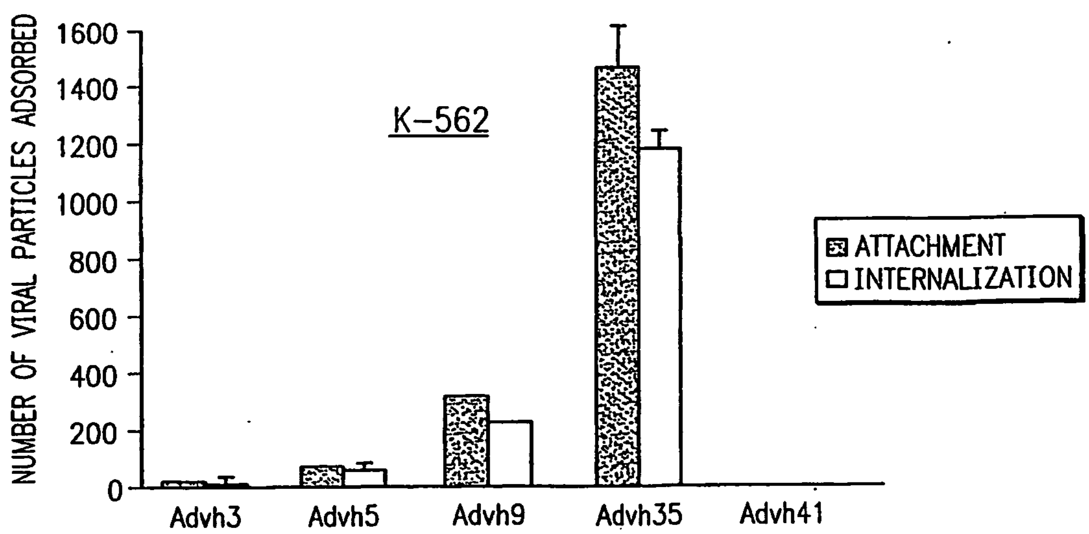


FIG. 14B

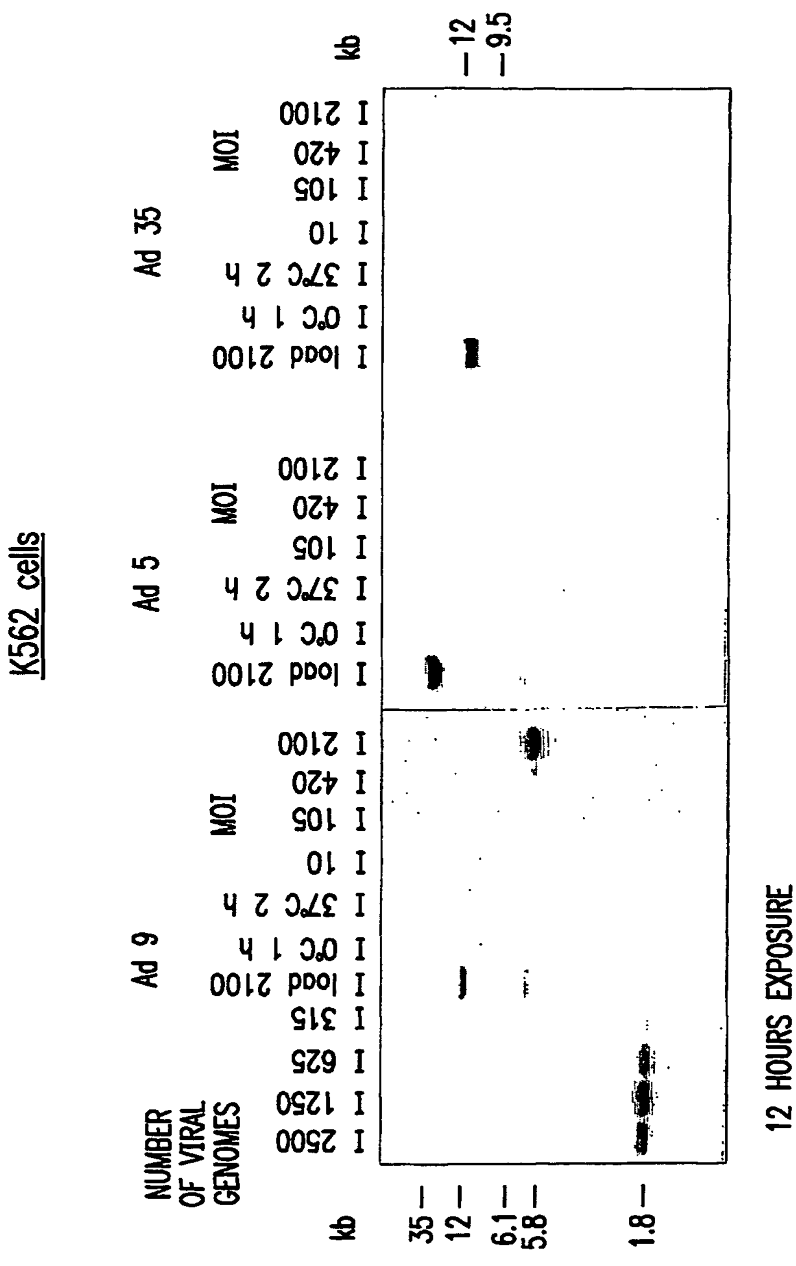

K562 cells
Ad 9
Ad 5
Ad 35
MOI
NUMBER OF VIRAL GENOMES
2500
1250
625
315
load 2100
0°C 1 h
37°C 2 h
10
105
420
2100
kb
35
12
6.1
5.8
1.8
9.5
12 HOURS EXPOSURE

FIG. 15A

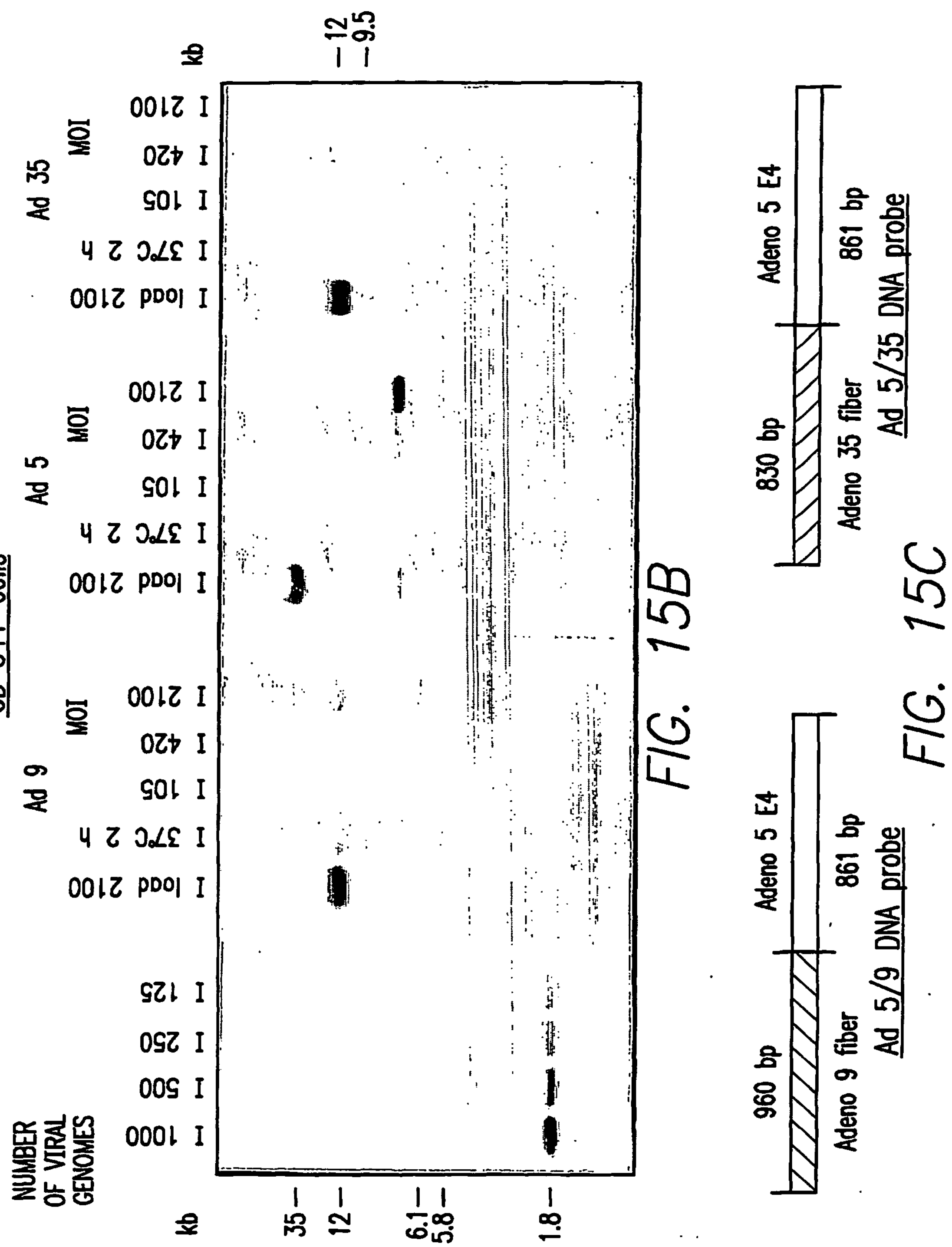


FIG. 15B

FIG. 15C

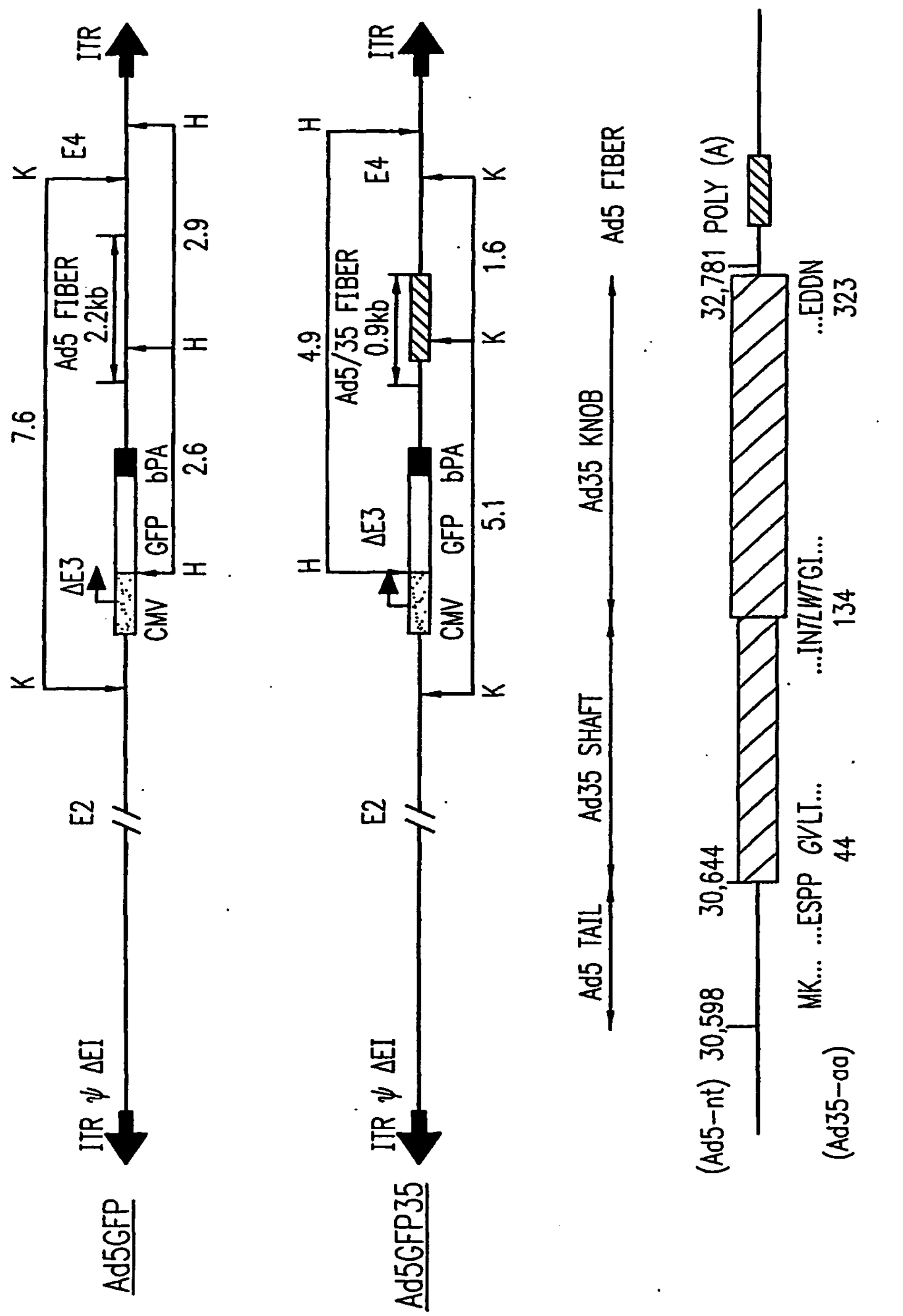

Ad5GFP
ITR ψ ΔEI
E2
K
CMV
ΔE3
GFP
bPA
7.6
Ad5 FIBER
2.2kb
H
2.6
2.9
E4
ITR
Ad5GFP35
Ad5/35 FIBER
0.9kb
4.9
5.1
1.6
Ad5 TAIL
Ad35 SHAFT
Ad35 KNOB
Ad5 FIBER
(Ad5-nt)
30,598
30,644
32,781
POLY (A)
(Ad35-aa)
MK...
...ESPP GVLT...
44
...INLWTGI...
134
...EDDN
323

FIG. 16A

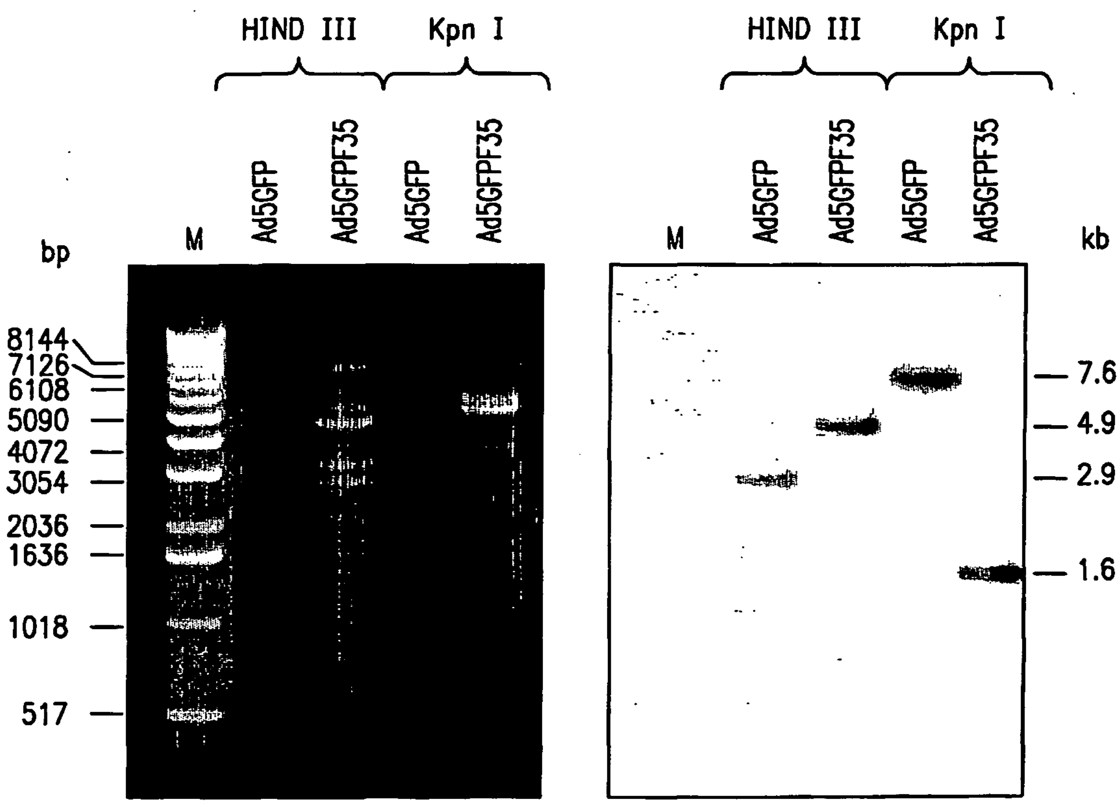

HIND III
Kpn I
M
Ad5GFP
Ad5GFPF35
Ad5GFP
Ad5GFPF35
bp
8144
7126
6108
5090
4072
3054
2036
1636
1018
517
HIND III
Kpn I
M
Ad5GFP
Ad5GFPF35
Ad5GFP
Ad5GFPF35
kb
7.6
4.9
2.9
1.6

FIG. 16B

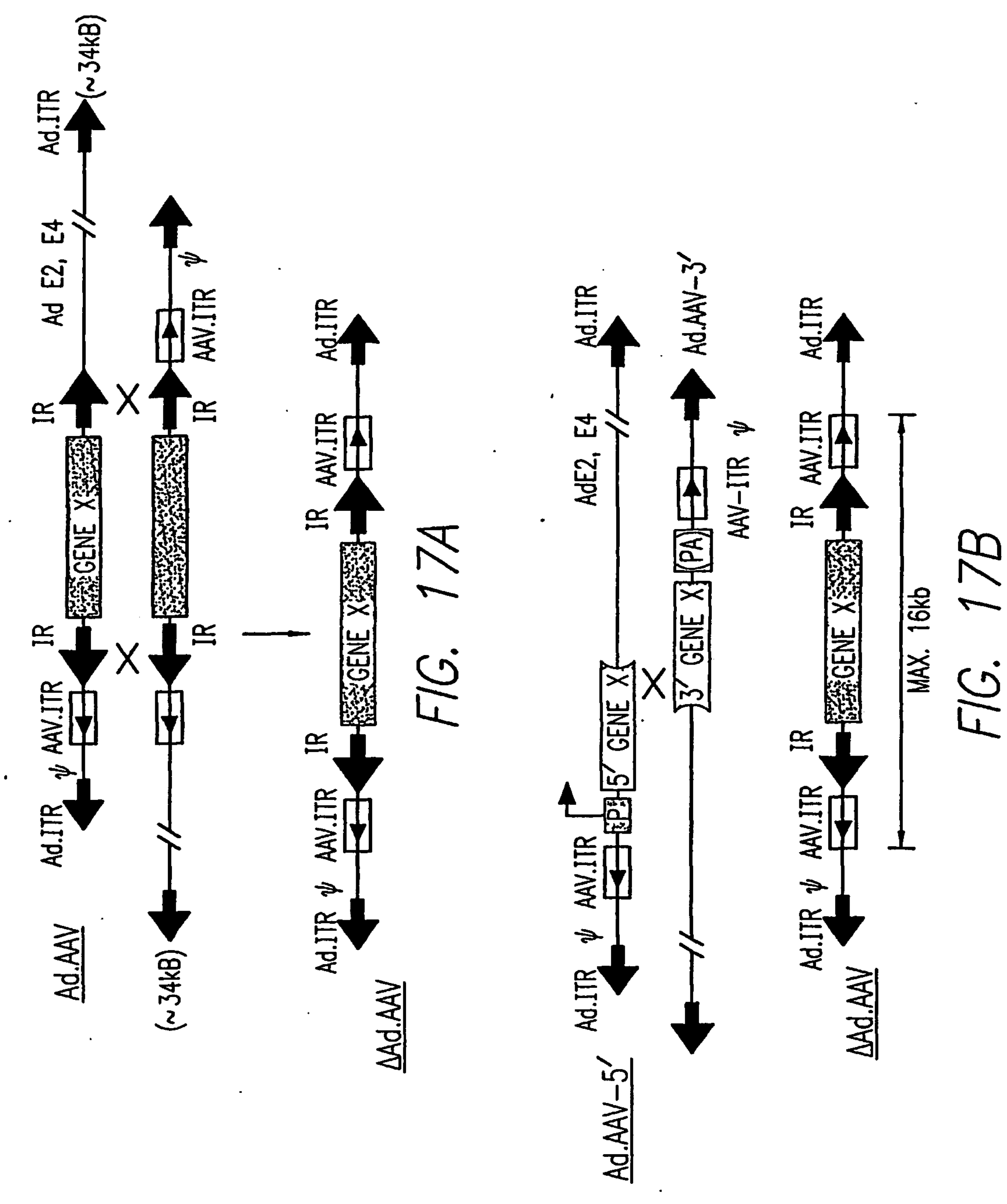
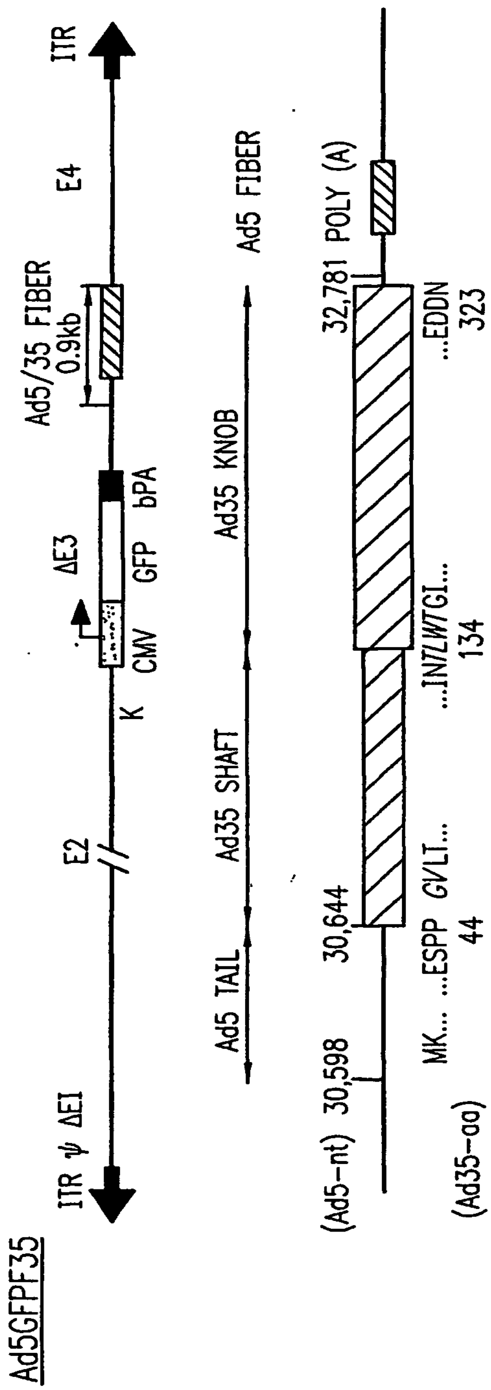


FIG. 17A

FIG. 17B



Ad5GFPF35
ITR ψ ΔEI
E2
K
CMV
GFP
bPA
ΔE3
Ad5/35 FIBER
0.9kb
E4
ITR
Ad5 TAIL
Ad35 SHAFT
Ad35 KNOB
Ad5 FIBER
(Ad5-nt)
30,598
30,644
32,781
POLY (A)
(Ad35-aa)
MK...
...ESPP GVLT...
44
...INTLWTGI...
134
...EDDN
323

FIG. 18

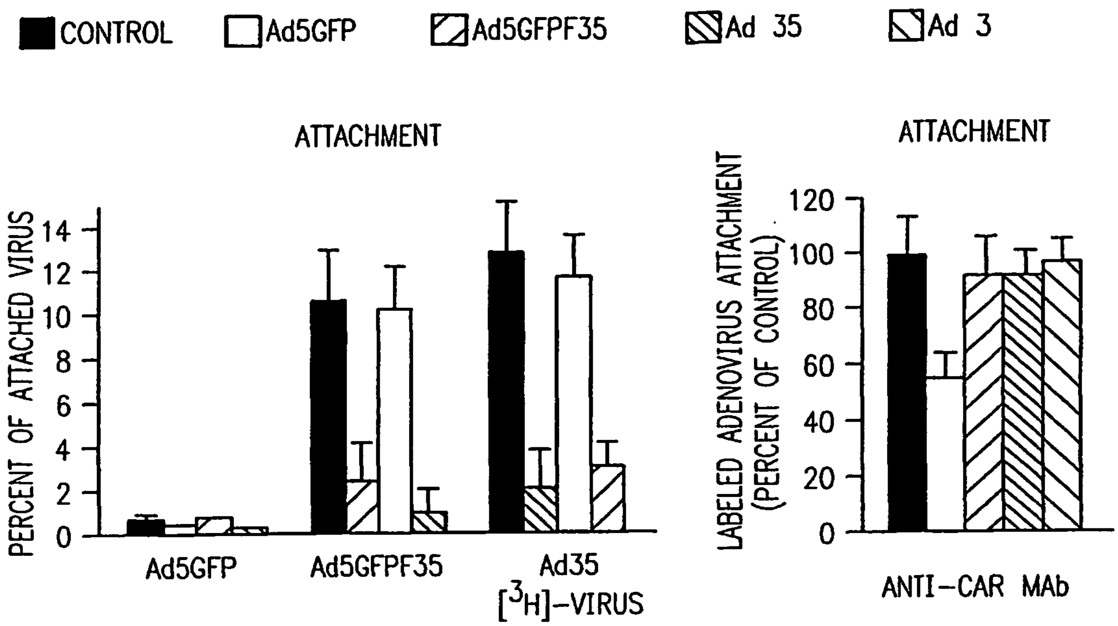


FIG. 19A FIG. 19B

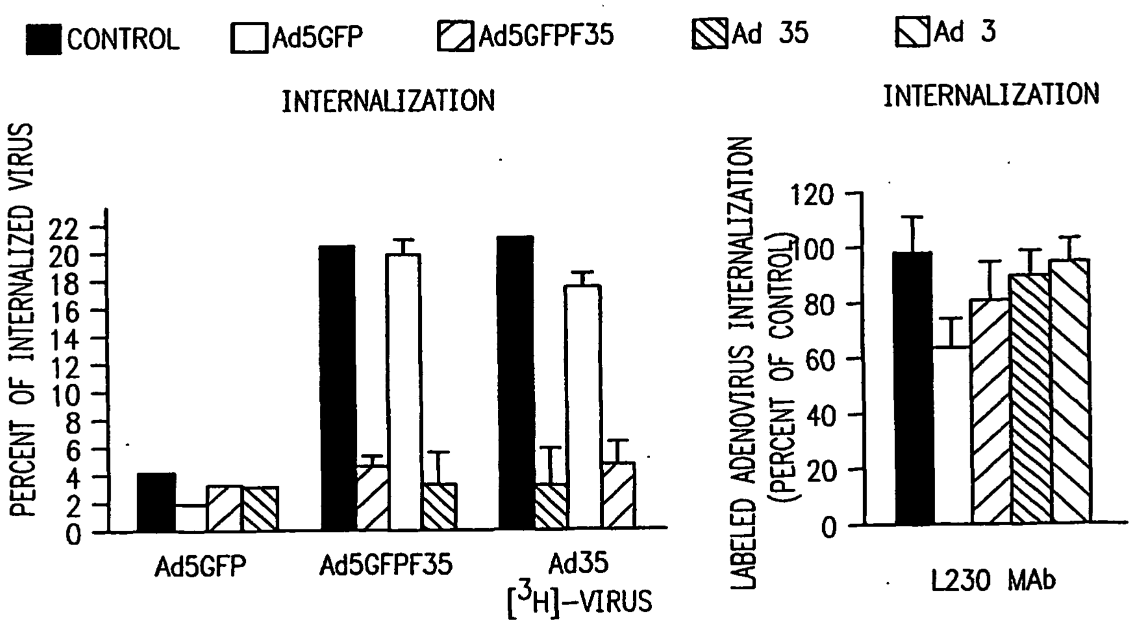


FIG. 19C FIG. 19D

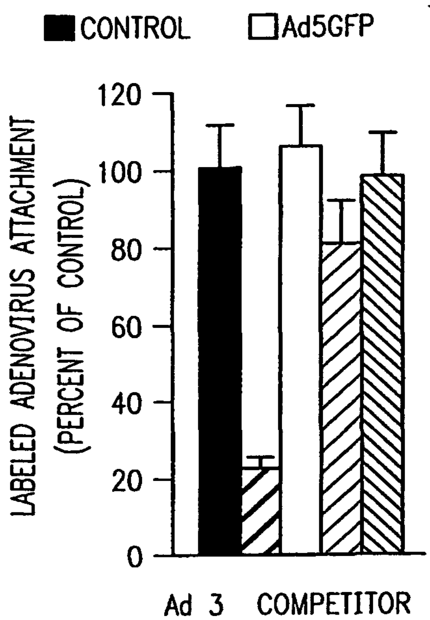


FIG. 20A

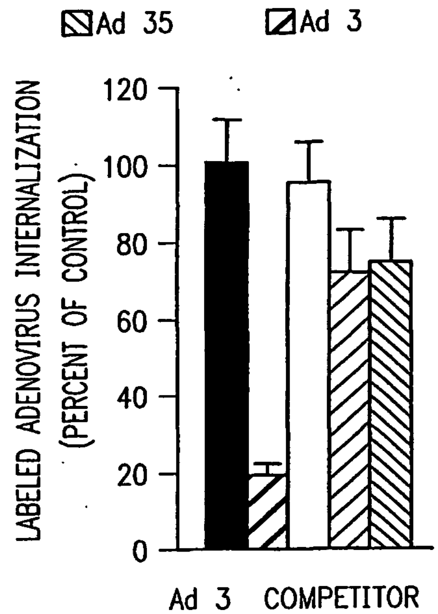


FIG. 20B

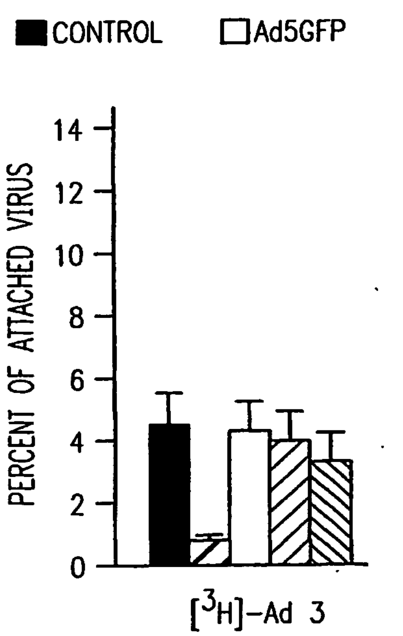


FIG. 20C

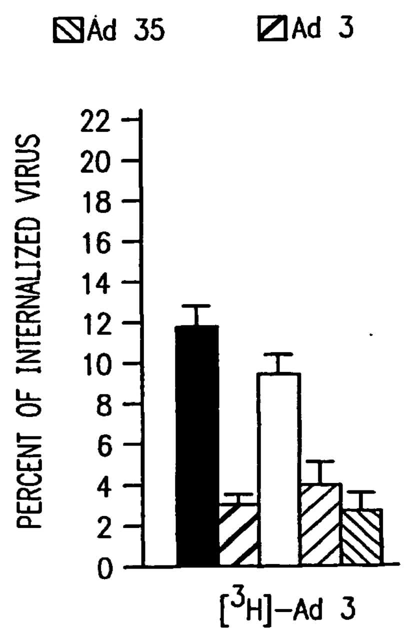


FIG. 20D

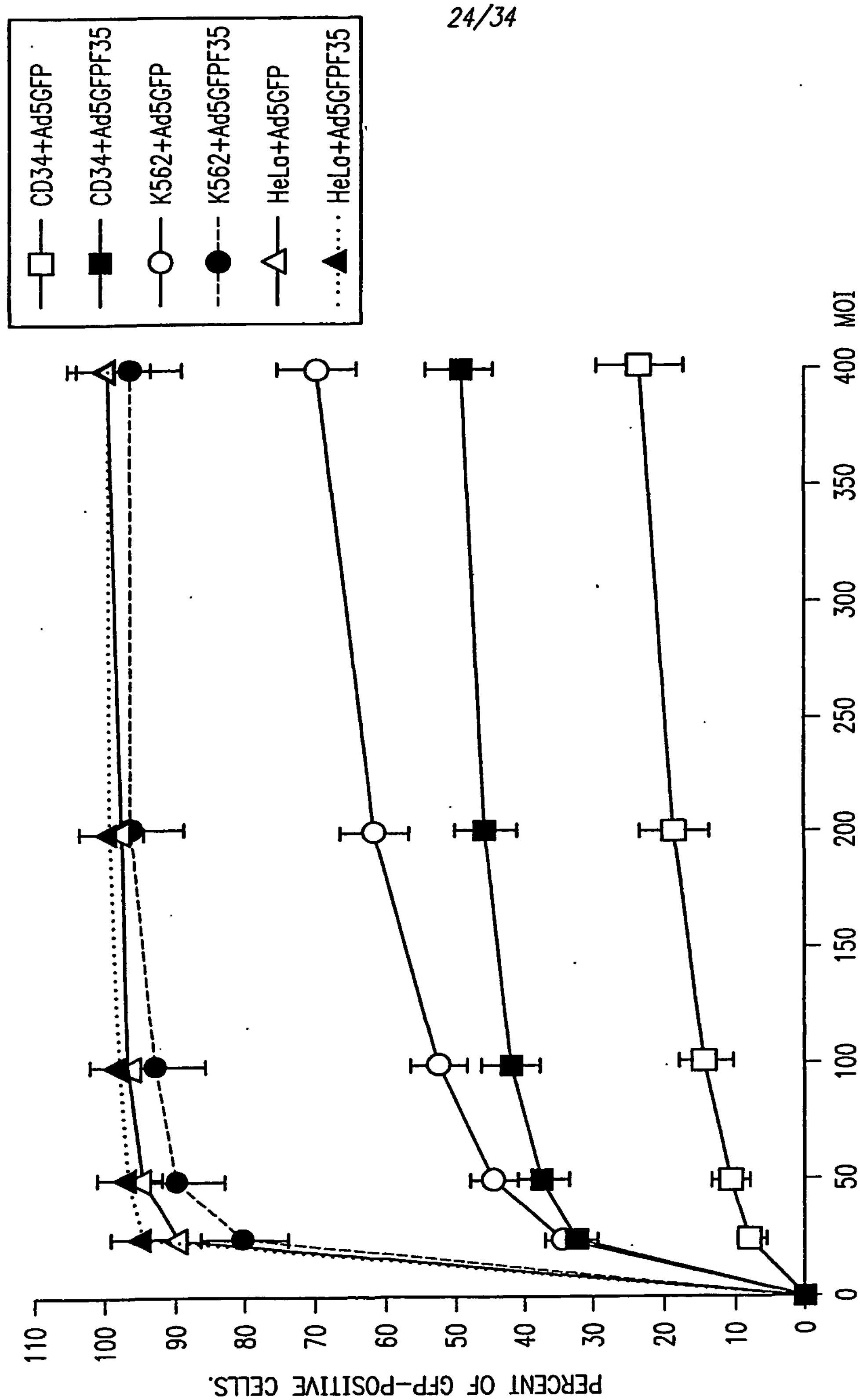

CD34+Ad5GFP
CD34+Ad5GFPF35
K562+Ad5GFP
K562+Ad5GFPF35
HeLa+Ad5GFP
HeLa+Ad5GFPF35
PERCENT OF GFP-POSITIVE CELLS.
0
10
20
30
40
50
60
70
80
90
100
110
0
50
100
150
200
250
300
350
400
MOI

FIG. 21

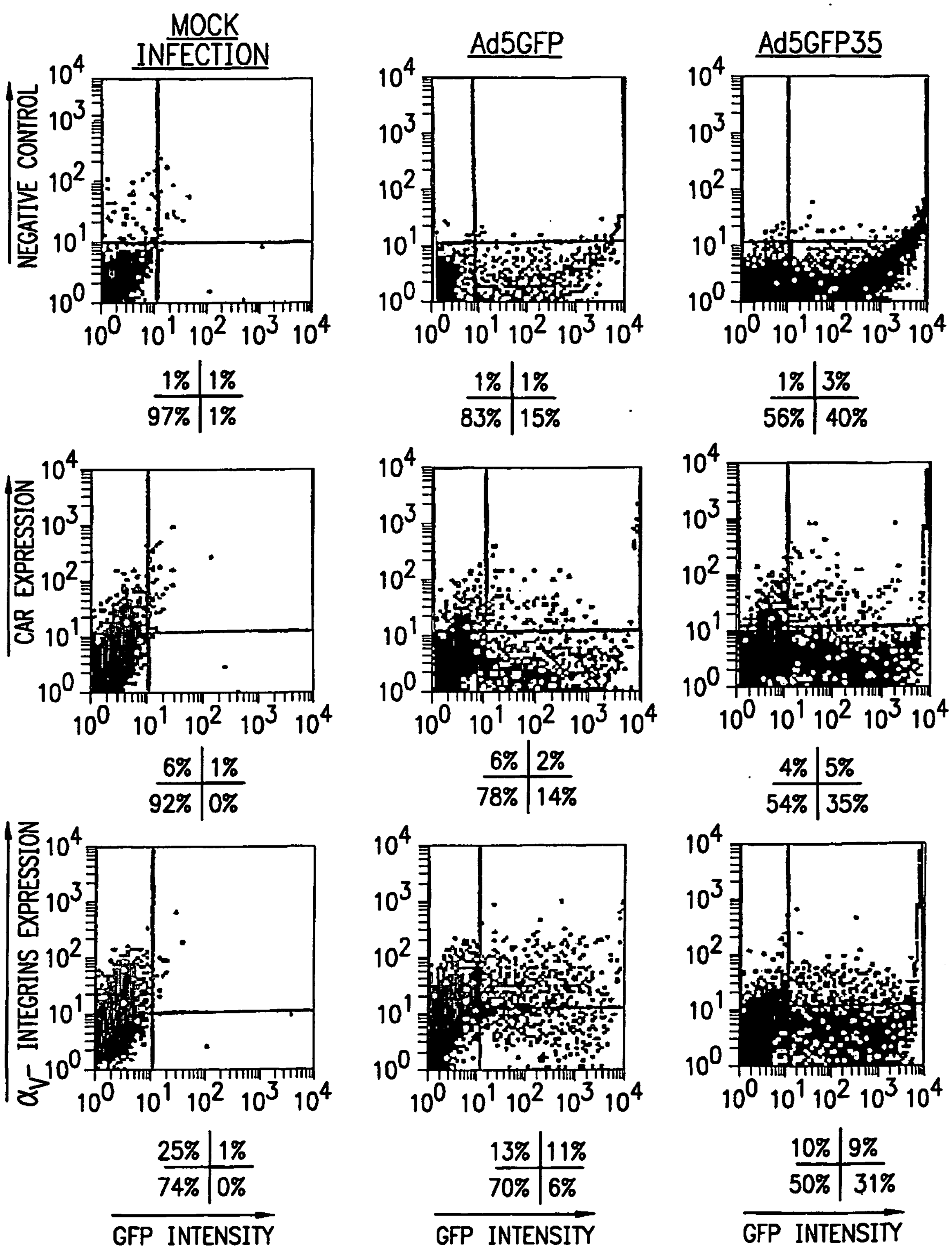

MOCK INFECTION
Ad5GFP
Ad5GFP35
NEGATIVE CONTROL
CAR EXPRESSION
α_V INTEGRINS EXPRESSION
10^0 10^1 10^2 10^3 10^4
1% 1% 97% 1%
1% 1% 83% 15%
1% 3% 56% 40%
6% 1% 92% 0%
6% 2% 78% 14%
4% 5% 54% 35%
25% 1% 74% 0%
13% 11% 70% 6%
10% 9% 50% 31%
GFP INTENSITY
GFP INTENSITY
GFP INTENSITY

FIG. 22

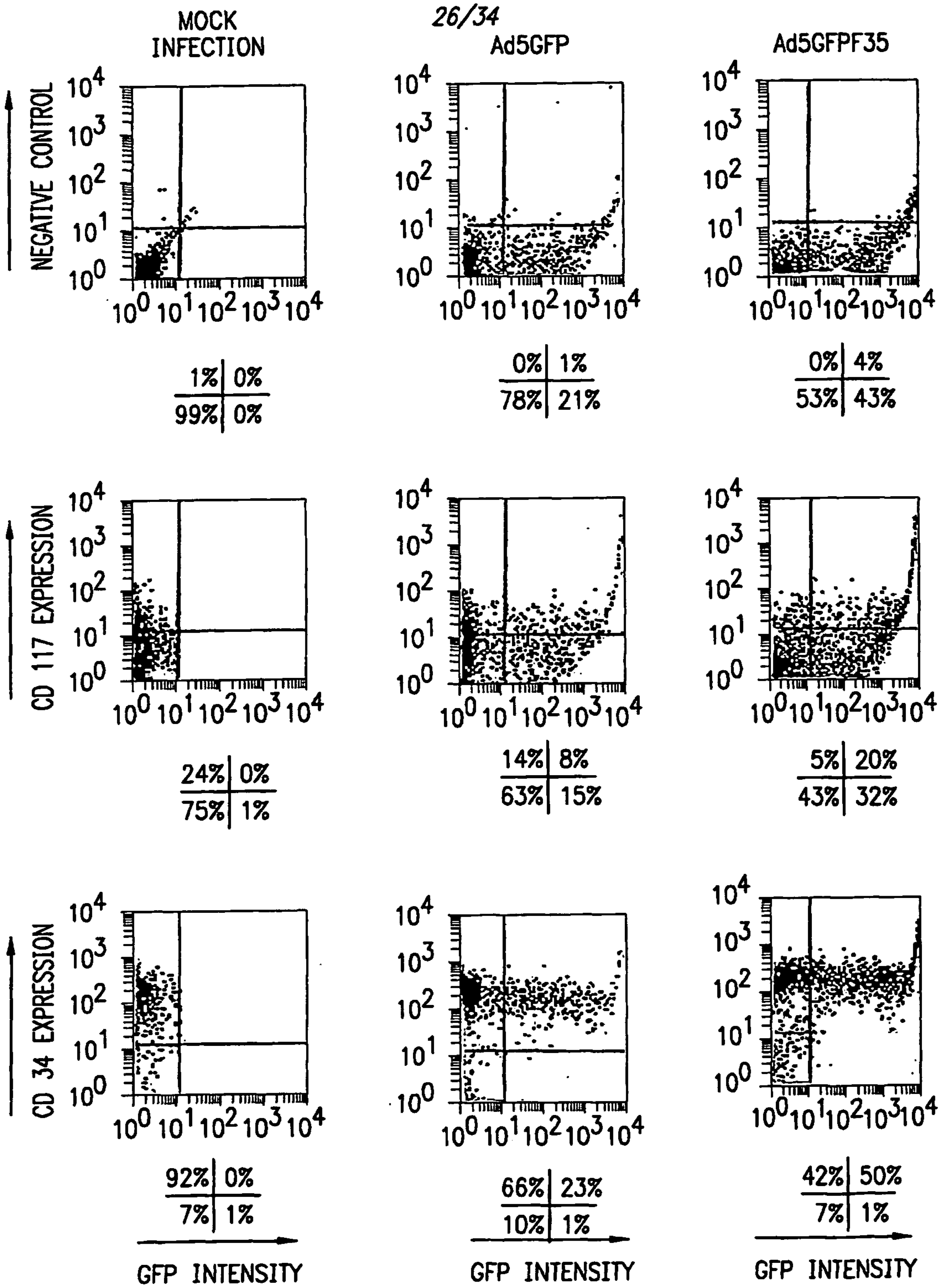

MOCK INFECTION
Ad5GFP
Ad5GFPF35
NEGATIVE CONTROL
CD 117 EXPRESSION
CD 34 EXPRESSION
10^0 10^1 10^2 10^3 10^4
1% | 0%
99% | 0%
0% | 1%
78% | 21%
0% | 4%
53% | 43%
24% | 0%
75% | 1%
14% | 8%
63% | 15%
5% | 20%
43% | 32%
92% | 0%
7% | 1%
66% | 23%
10% | 1%
42% | 50%
7% | 1%
GFP INTENSITY
GFP INTENSITY
GFP INTENSITY

FIG. 23A

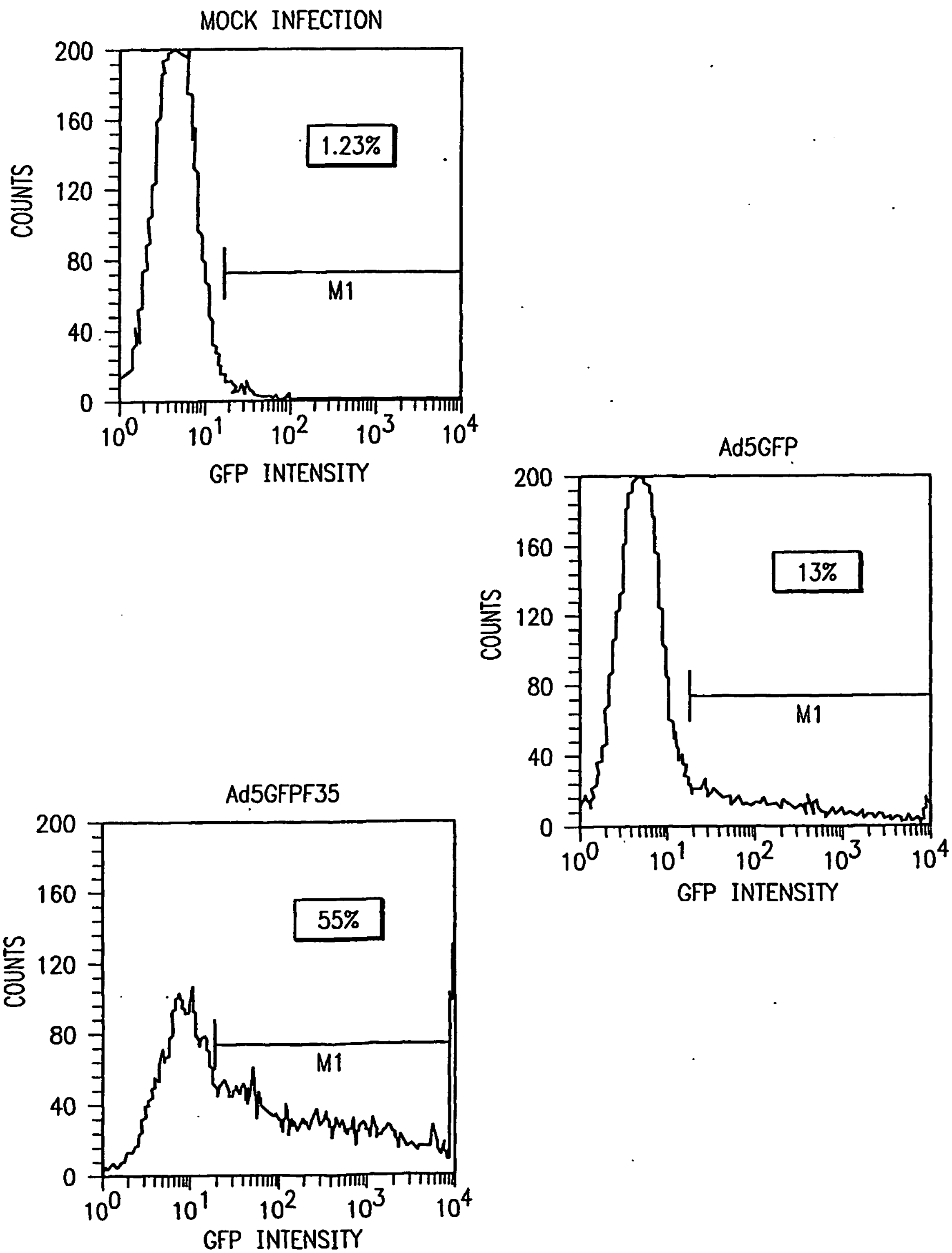

MOCK INFECTION
200
160
120
80
40
0
COUNTS
1.23%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY
Ad5GFP
200
160
120
80
40
0
COUNTS
13%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY
Ad5GFPF35
200
160
120
80
40
0
COUNTS
55%
M1
10^0 10^1 10^2 10^3 10^4
GFP INTENSITY

FIG. 23B

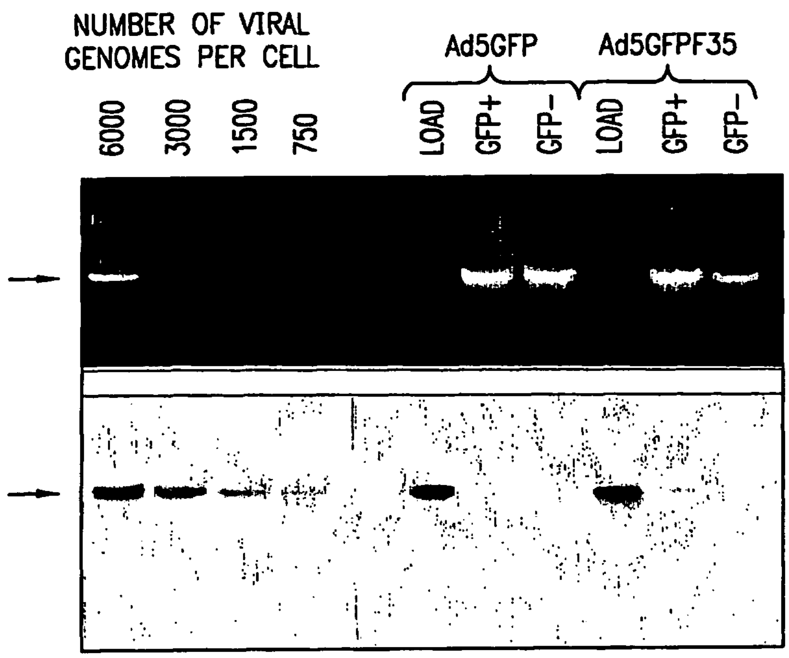


FIG. 24A

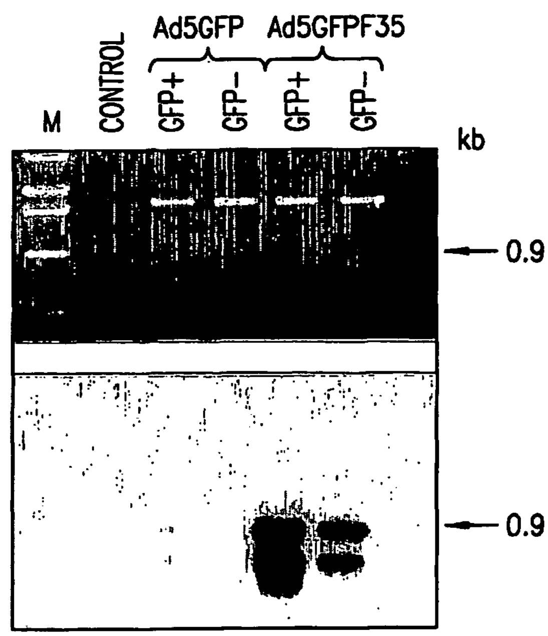
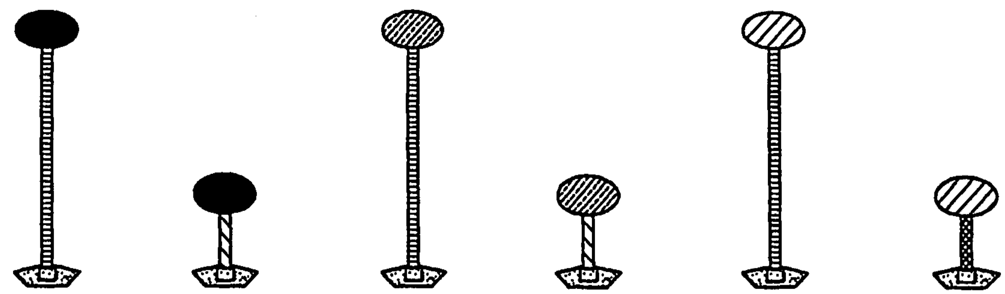


FIG. 24B

STRUCTURE AND FEATURES OF CHIMERIC ADENOVIRUS VECTORS.



Adeno vector	Ad 5L	Ad 5S	Ad 5/9L	Ad 5/9S	Ad 5/35L	Ad 5/35S
Binding to CAR	YES	YES	YES	YES	NO	NO
genome/PFU ratio	13	143	29	582	17	14
Attachment to/internalization into						
293	+++	++	++	+/--	+++	+++
K562	-/+	--/+	--/+	---	+++	+++
Y79	++	+	+	+/-	+	+
Gene transfer efficiency						
293	+++	++	++	+/--	+++	+++
K562	-/+	--/+	--/+	---	+++	+++
Y79	++	+	+	+/-	+	+

FIG. 25

Top View of Adenovirus Type 5 Fiber Knob Domain

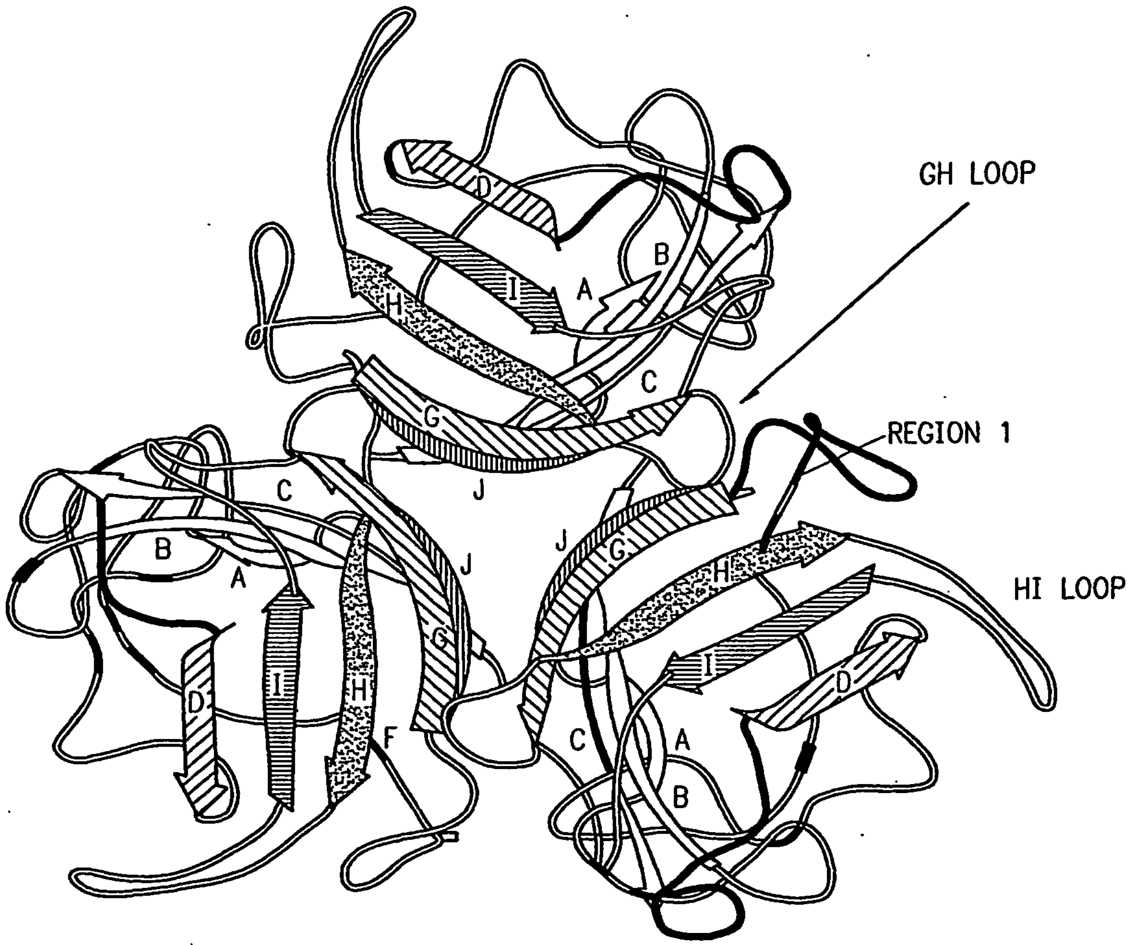


FIG. 26

Amino Acid Composition of Wt and Modified Adeno 5 GH-loop

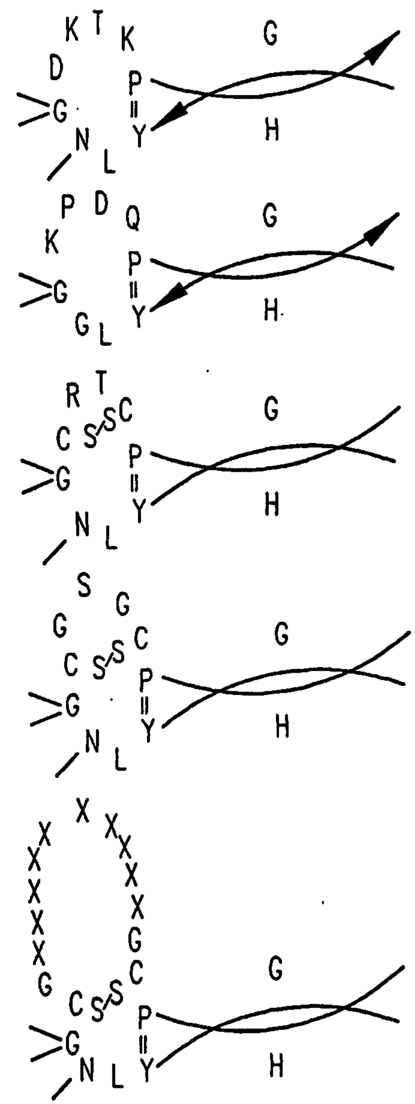
Adeno 5 wt GH-loop	V Y]L N G D K T K [P V	
Adeno 5/9 GH-loop chimera	V Y]L G G K P D Q [P V	
Adeno 5 GH-Cys 1	V Y]L N G C G S C [P V	
Adeno 5 GH-Cys 2	V Y]L N G C G S G C [P V	
Adeno 5 GH-peptide	V Y]L N G C G $(X)^{10}$ G C [P V	

FIG. 27

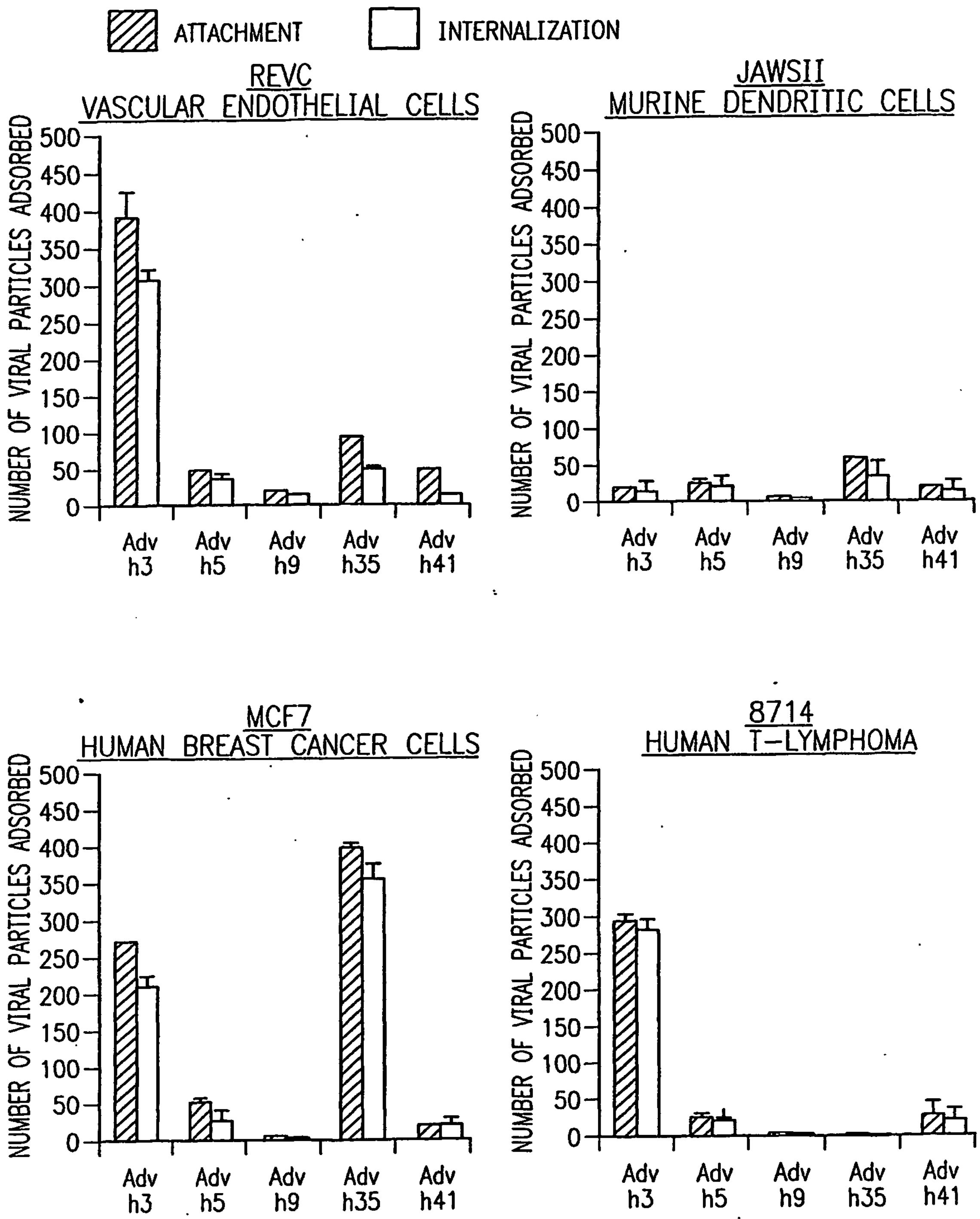

ATTACHMENT
INTERNALIZATION
REVC
VASCULAR ENDOTHELIAL CELLS
JAWSII
MURINE DENDRITIC CELLS
MCF7
HUMAN BREAST CANCER CELLS
8714
HUMAN T-LYMPHOMA
NUMBER OF VIRAL PARTICLES ADSORBED
500
450
400
350
300
250
200
150
100
50
0
Adv h3
Adv h5
Adv h9
Adv h35
Adv h41

FIG. 28

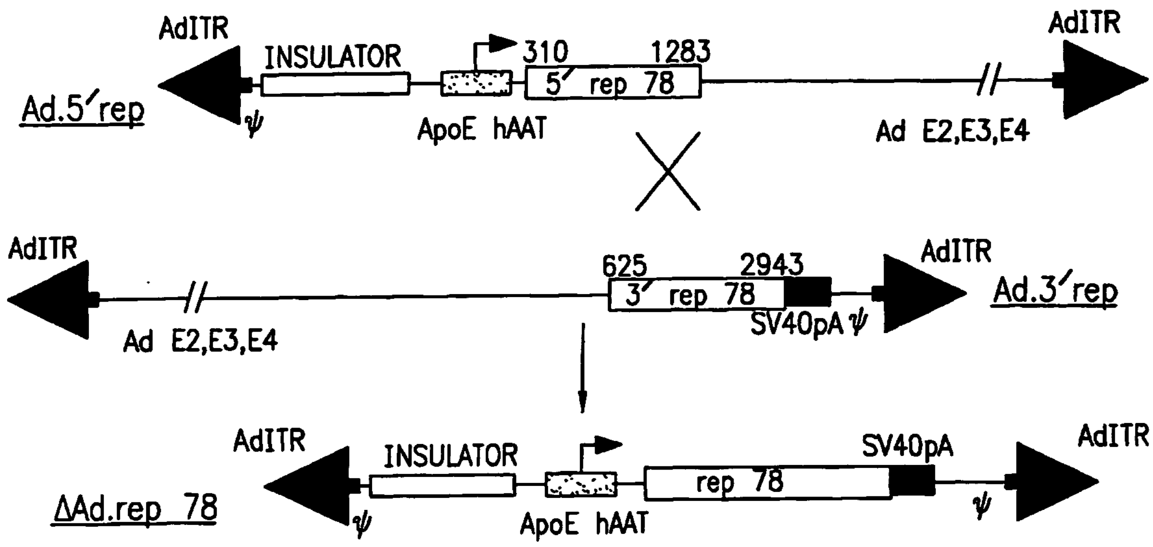


FIG. 29A

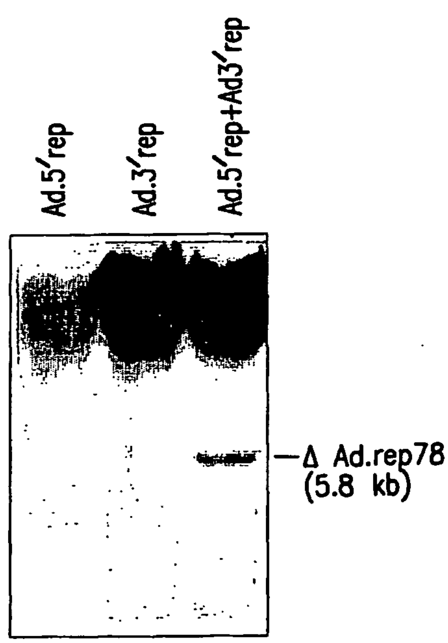


FIG. 29B

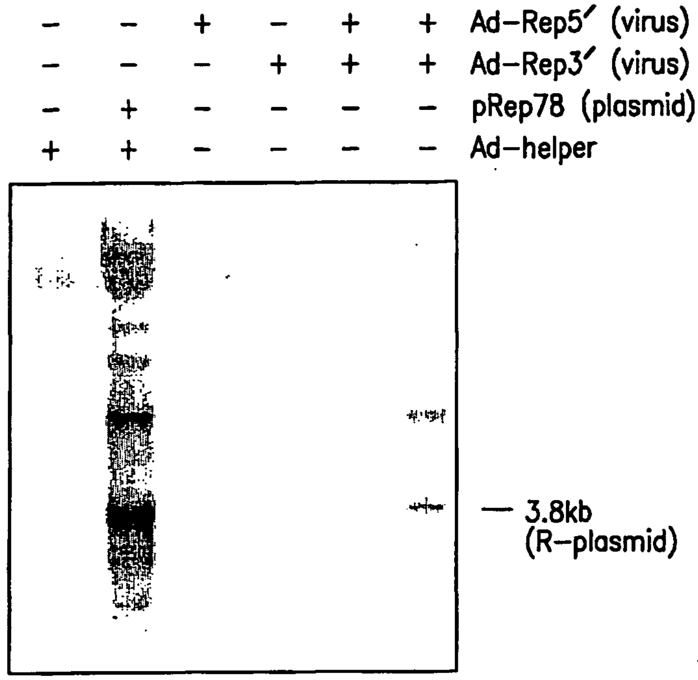


FIG. 29C

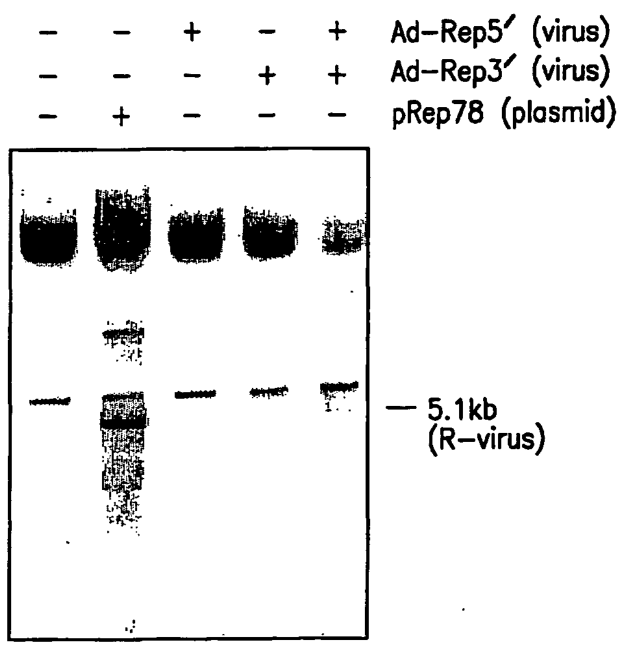


FIG. 29D

INTERNATIONAL SEARCH REPORT

Intern   ıal Application No
PCT/US 00/15442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/861 C12N15/864 C12N15/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 856 152 A (WILSON JAMES M ET AL) 5 January 1999 (1999-01-05)	1,2, 5-11,15, 36-44, 48-54,58
Y	the whole document	3,4,13, 16-32, 35,36, 46,47
Y	WO 98 54346 A (ROELVINK PETRUS W ;EINFELD DAVID (US); GENVEC INC (US); KOVESDI IM) 3 December 1998 (1998-12-03) page 7, line 27 -page 14, line 26 the whole document	3,4, 16-32, 35,56

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2000	30/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 00/15442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	BURCIN M. M. ET AL.: "Adenovirus-mediated regulable target gene expression in vivo" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 96, January 1999 (1999-01), pages 355-360, XP002130326 ISSN: 0027-8424	1,2
Y	the whole document	13,46
X	RECCHIA A. ET AL.: "Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 6, 16 March 1999 (1999-03-16), pages 2615-2620, XP002152917 ISSN: 0027-8424 the whole document	1,2, 5-11,14, 36-44, 47-49,58
X	WO 97 38723 A (BARBER JACK ;IMMUSOL INC (US); LI QI XIANG (US); YU GANG (US); YU) 23 October 1997 (1997-10-23)	55,57
Y	page 4, line 14 - line 20 page 5, line 21 -page 6, line 10 page 29, line 4 -page 30, line 2 page 35, line 12 - line 18	56
X	WO 94 06920 A (MEDICAL RES COUNCIL ;RUSSELL STEPHEN JAMES (GB); HAWKINS ROBERT ED) 31 March 1994 (1994-03-31) page 19, last paragraph	55,57
A	GALL J. ET AL.: "ADENOVIRUS TYPE 5 AND 7 CAPSID CHIMERA: FIBER REPLACEMENT ALTERS RECEPTOR TROPISM WITHOUT AFFECTING PRIMARY IMMUNE NEUTRALIZATION EPITOPES" JOURNAL OF VIROLOGY, vol. 70, no. 4, 1 April 1996 (1996-04-01), pages 2116-2123, XP000653519 ISSN: 0022-538X the whole document	16-25
A	LYU Y. L. ET ALW: "Inversion/dimerization of plasmids mediated by inverted repeats." JOURNAL OF MOLECULAR BIOLOGY, vol. 285, no. 4, 29 January 1999 (1999-01-29), pages 1485-1501, XP002152918 ISSN: 0022-2836 cited in the application the whole document	1-58

-/--

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 00/15442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,Y	STEINWAERDER D. S. ET AL.: "Generation of adenovirus vectors devoid of all viral genes by recombination between inverted repeats." JOURNAL OF VIROLOGY, vol. 73, no. 11, November 1999 (1999-11), pages 9303-9313, XP002152919 ISSN: 0022-538X the whole document ---	1-58
P,Y	LIEBER A. ET AL.: "Integrating adenovirus-adeno-associated virus hybrid vectors devoid of all viral genes." JOURNAL OF VIROLOGY, vol. 73, no. 11, November 1999 (1999-11), pages 9314-9324, XP002152920 ISSN: 0022-538X the whole document ----	1-58

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Interr nal Application No
PCT/US 00/15442

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 5856152	A	05-01-1999	AU	695811	B	20-08-1998
			AU	4405596	A	23-05-1996
			CA	2203808	A	09-05-1996
			EP	1046711	A	25-10-2000
			EP	0797678	A	01-10-1997
			JP	10507928	T	04-08-1998
			WO	9613598	A	09-05-1996
			US	5871982	A	16-02-1999
WO 9854346	A	03-12-1998	AU	7604998	A	30-12-1998
			BR	9809173	A	01-08-2000
			EP	0988390	A	29-03-2000
			PL	337130	A	31-07-2000
WO 9738723	A	23-10-1997	AU	2678097	A	07-11-1997
			CA	2251738	A	23-10-1997
			EP	0927044	A	07-07-1999
WO 9406920	A	31-03-1994	AU	689755	B	09-04-1998
			AU	4827893	A	12-04-1994
			CA	2145063	A	31-03-1994
			EP	0670905	A	13-09-1995
			JP	8504091	T	07-05-1996
			US	5723287	A	03-03-1998

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 November 2001 (08.11.2001)

PCT

(10) International Publication Number
WO 01/83729 A2

(51) International Patent Classification[7]: C12N 15/00

(21) International Application Number: PCT/EP01/04863

(22) International Filing Date: 30 April 2001 (30.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/562,934 1 May 2000 (01.05.2000) US

(71) Applicants: **NOVARTIS AG** [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH). **THE SCRIPPS RESEARCH INSTITUTE** [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).

(71) Applicants and
(72) Inventors: **NEMEROW, Glen, R.** [US/US]; 462 Cerro Street, Encinitas, CA 92024 (US). **VON SEGGERN, Daniel, J.** [US/US]; Apartment 30, 5175 Luigi Terrace, San Diego, CA 92122 (US). **FRIEDLANDER, Marty** [US/US]; 1720 Zapo Street, Del Mar, CA 92014 (US).

(74) Agent: **BECKER, Konrad**; Novartis AG, Corporate Intellectual Property, Patent & Trademark Dept., CH-4002 Basel (CH).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/83729 A2

(54) Title: VECTORS FOR OCULAR TRANSDUCTION AND USE THEREOF FOR GENETIC THERAPY

(57) Abstract: Adenovirus vector-based gene therapy methods for treating ocular disorders are provided. Adenovirus vectors for therapy of ocular diseases and methods of treatment using the vectors are provided. Compositions, kits, and methods of preparation and use of the vectors for gene therapy are provided.

VECTORS FOR OCULAR TRANSDUCTION AND USE THEREOF FOR GENETIC THERAPY

Work described herein was supported by NIH grants EY11431 and HL54352. The government has certain rights in such subject matter.

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. application Serial No. 09/562,934, filed May 1, 2000, to Glen R. Nemerow, Daniel Von Seggern,; Martin Friedlander, entitled "VECTORS FOR OCULAR TRANSDUCTION AND USE THEREFOR FOR GENETIC THERAPY".

This application is related to copending U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265, filed January 14, 2000)), to Daniel Von Seggern, Glen R. Nemerow, Paul Hallenbeck, Susan Stevenson, Yelena Skripchenko, filed January 14, 2000, entitled "Adenovirus Vectors, Packaging Cell Lines,Compositions, and Methods for Preparation and Use," which is a continuation-in-part of U.S. Application 09/423,783 filed November 12, 1999 and claims the benefit of the filing date of U.S. Provisional Application 60/115,920 filed January 14, 1999. Where permitted, the contents and subject matter of each application and of the provisional application are incorporated in their entirety herein by reference.

FIELD OF INVENTION

The present invention relates to gene therapy, especially to adenovirus vector-based gene therapy. In particular, adenovirus vectors for therapy of ocular diseases and methods of treatment using the vectors are provided. Compositions, kits, and methods of preparation and use of the vectors for gene therapy are provided.

BACKGROUND OF THE INVENTION

Retinal dystrophies

The eye is susceptible to a number of hereditary and/or age related degenerative disorders. In the United States, common causes of irreversible blindness or severe loss of vision are retinal dystrophies (see, *e.g.*, Cotlier *et al.* (1995) *Surv. Ophthalmology 40*:51-61; Bird (1995) *Am. J. Ophthal. 119*: 543-562; and Adler (1996) *Arch Ophthal 114*:79-83). The retina is the sensory

tunic of the eye, containing light sensitive receptors, a complex of neurons, and pigmented epithelium, arranged in discrete layers. In humans, the macula is the portion of the retina that lies directly behind the lens. Cones, the photoreceptor cells responsible for central vision, are heavily concentrated in the macula. Central dystrophies, which affect the macula, include Best's disease, age-related macular degeneration, and Stargardt's macular dystrophy. The peripheral retina is composed mainly of rods, which are responsible for side and night vision. Peripheral degenerative retinal diseases include retinitis pigmentosa, choroidemia and Bietti's crystalline dystrophy.

Macular degenerations are a heterogenous group of diseases, characterized by progressive central vision loss and degeneration of the macula and underlying retinal pigmented epithelium. Age-related macular degeneration (ARMD) is the most common form of the disease, affecting an estimated 20% of persons over 75 years of age. ARMD is poorly understood in terms of etiology and pathogenesis. The very late onset of the disease has made genetic mapping particularly difficult. Certain macular degenerative conditions with a clear genetic basis, such as Stargardt's and Best's diseases, share many features with ARMD, but have been more amenable to molecular and genetic analysis.

Hereditary peripheral retinopathies are also relatively common. Retinitis pigmentosa (RP), for example, affects approximately 1.5 million people worldwide. Substantial genetic heterogeneity has been observed in this condition, with over 20 chromosomal loci identified. A predisposition to retinitis pigmentosa can be inherited by autosomal dominant, autosomal recessive, X-linked or digenic mode. Mutations have been identified in seven genes, four of which encode proteins in the rod phototransduction cascade: rhodopsin, alpha and beta subunits of rod cGMP phosphodiesterase, and rod cGMP cation-gated channel protein .alpha. subunit. Mutations in the peripherin/RDS gene have been linked to retinitis pigmentosa and macular degeneration. A single peripherin/RDS mutation apparently caused retinitis pigmentosa, pattern dystrophy and fundus flavimaculatus, in different family members.

In spite of causal heterogeneity, there is significant clinical similarity among RP subtypes. Common signs and symptoms include early electroretinographic abnormalities, ophthalmoscopic findings, and protracted, contiguous expansion of the ring-like scotoma toward the macula, leading to progressively worsening tunnel vision. A recent hypothesis is that active photoreceptor cell death, which is characteristic of these genetically distinct disorders, is mediated by a common induction of apoptosis. It may be possible to treat these conditions by the administration of agents that block induction of apoptosis in photoreceptors, such as neurotrophic factors.

Adenovirus delivery vectors

Adenovirus, which is a DNA virus with a 36 kilobase (kb) genome, is very well-characterized and its genetics and genetic organization are understood. The genetic organization of adenoviruses permits substitution of large fragments of viral DNA with foreign DNA. In addition, recombinant adenoviruses are structurally stable and no rearranged viruses are observed after extensive amplification.

Adenoviruses have been employed as delivery vehicles for introducing desired genes into eukaryotic cells. The adenovirus delivers such genes to eukaryotic cells by binding to cellular receptors followed by internalization. The adenovirus fiber protein is responsible for binding to cells. The fiber protein has two domains, a rod-like shaft portion and a globular head portion that contains the receptor binding region. The fiber spike is a homotrimer, and there are 12 spikes per virion. Human adenoviruses bind to and infect a broad range of cultured cell lines and primary tissues from different species.

The 35,000+ base pair (bp) genome of adenovirus type 2 has been sequenced and the predicted amino acid sequences of the major coat proteins (hexon, fiber and penton base) have been described (see, *e.g.*, Neumann *et al.*, *Gene 69*: 153-157 (1988); Herisse *et al.*, *Nuc. Acids Res. 9*: 4023-4041 (1981); Roberts *et al.*, *J. Biol. Chem. 259*: 13968-13975 (1984); Kinloch *et al.*, *J. Biol. Chem. 259*: 6431-6436 (1984); and Chroboczek *et al.*, *Virol. 161*: 549-554, 1987).

The 35,935 bp sequence of Ad5 DNA is also known and portions of many other adenovirus genomes have been sequenced. The upper packaging limit for adenovirus virions is about 105% of the wild-type genome length (see, *e.g.*, Bett, *et al., J. Virol. 67(10):* 5911-21, 1993). Thus, for Ad2 and Ad5, this would be an upper packaging limit of about 38kb of DNA.

Adenovirus DNA also includes inverted terminal repeat sequences (ITRs) ranging in size from about 100 to 150 bp, depending on the serotype. The inverted repeats permit single strands of viral DNA to circularize by base-pairing of their terminal sequences to form base-paired "panhandle" structures that are required for replication of the viral DNA.
For efficient packaging, the ITRs and the packaging signal (a few hundred bp in length) comprise the "minimum requirement" for replication and packaging of a genomic nucleic acid into an adenovirus particle. Helper-dependent vectors lacking all viral ORFs but including these essential *cis* elements (the ITRs and contiguous packaging sequence) have been constructed.

Ad vectors have several distinct advantages as gene delivery vehicles. For example, recombination of such vectors is rare; there are no known associations of human malignancies with adenoviral infections despite common human infection with adenoviruses; the genome may be manipulated to accommodate foreign genes of a fairly substantial size; and host proliferation is not required for expression of adenoviral proteins. Adenovirus (Ad)-based gene delivery vectors efficiently infect many different cells and tissues. This broad tropism, however, means that gene delivery cannot be directed to a specific target cell. A large fraction of intravenously administered adenovirus is retained by the liver, which could lead to undesirable side-effects. Adenovirus may potentiate immune responses. For example, Adenovirus type 5 (Ad5) also transduces dendritic cells, which present antigens very efficiently, thereby possibly exacerbating the immune response against the vector. It has been proposed that vectors with different targeting efficiencies might eliminate these problems, permitting a lower total particle dose and more specific targeting (see, *e.g.*, U.S. application Serial No. 09/482,682).

The wealth of information on adenovirus structure and mechanism of infection, its efficient infection of nondividing cells, and its large genetic capacity make adenovirus a popular gene therapy vector. The wide expression of receptors to which adenovirus binds makes targeting adenovirus vectors difficult.

Hence there is a need to improve delivery and targeting of adenoviral vectors and also to provide treatments for ocular disorders. Therefore, it is an object herein to provide adenoviral vectors that specifically or selectively target cells in the eye. It is also an object herein to provide these vectors for treatment of ocular disorders.

SUMMARY OF THE INVENTION

Degenerative ocular diseases, such as, but not limited to, retinitis pigmentosa, Stargardt's disease, diabetic retinopathies, retinal vascularization, and others (see, *e.g.*, Table below), have a genetic basis. Genes expressed in the photoreceptor cells at the back of the retina are implicated in these diseases. Provided herein are recombinant viral vectors for targeting therapeutic products to these cells.

Recombinant adenoviral vectors that include nucleic acid that permits specific binding to these photoreceptors are provided. In particular, the vector particles contain a fiber protein of Ad37 or a modified form thereof. As shown herein, fiber protein from Ad37 permits efficient infection of photoreceptor cells. Fiber proteins from other adenovirus D serotypes may also be used. In addition, the portions of the fiber protein, particularly those that interact with other viral structural proteins, such as penton, may be modified to resemble the viral source of the other structural proteins. As exemplified herein, the recombinant virus provided herein include Ad5 structural components. The N-terminus of the Ad37 fiber protein, which interacts with the penton protein, is modified to resemble the Ad5 fiber protein N-terminus to ensure production of viral particles.

The recombinant adenoviral vectors are intended for gene therapy of diseases in which genes expressed in the photoreceptors are implicated. Such diseases include, but are not limited to, degenerative ocular diseases, such as retinitis pigmentosa and Stargardt's disease. These vectors are also useful for

targeting to other ocular cells, such as conjunctival cells, which also bear receptors to which fiber from Ad37 and related serotypes bind.

The vectors will deliver therapeutic agents to the targeted cells for treatment of a variety of disorders (see *e.g.*, Tables 3 and 4, below)). The therapeutic agents are intended for expression in the photoreceptors and for secretion from the photorecptor cells, which are surrounded on one side by choroidal vasculature, and on the other side by retinal vasulature, thereby providing a means for delivery of products. In addition, expression of growth factors, such as VEGF and others, can be used to enhance blood flow to the retina and prevent or slow the degeneration.

Therapeutic agents encoded by the recombinant adenoviral vectors include, but are not limited to, nucleic acid nucleic acid molecules encoding genes that are defective in certain hereditary disorders, nucleic acid molecules that encode antiangiogenics and antitumor agents for treatment of retinal disorders, such as retinoblastomas; nucleic acid molecules encoding trophic factors, such as glial cell line-derived neuroptrophic factor (GDNF) and ciliary neurotrophic factor (CNTF), growth factors and growth factor inhibitors, antiapoptotic factors, such as Bcl-2 (CNTF), antitumor agents, anti-angiogenics, and genes or portions thereof for gene replacement or repair of defective genes. Hence, methods for treatment of inherited and acquired retinal diseases, including diseases involving neovascular and vascular degeneration are provided.

Methods for treating diseases involving genes expressed in photoreceptor cells are provided herein. The methods provided herein are practiced by administration of the recombinant viral vectors by any means suitable for delivery to the photoreceptors. A preferred mode of administration is intraocular injection including intravitreal and subretinal injection. Other modes of administration include, but are not limited to, intrascleral, periorbital and intravenous administration. The vectors also can include photoreceptor-specific promoters thereby providing a means, not only for specific targeting of expression in these cells, but also for photoreceptor-restricted transgene expression.

DETAILED DESCRIPTION OF THE INVENTION

A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to anywhere in the disclosure herein are incorporated by reference in their entirety.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art (see, Table 1).

As used herein, amino acid residue refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem., 243*:3552-59 (1969) and adopted at 37 C.F.R. § § 1.821 - 1.822, abbreviations for amino acid residues are shown in the following Table:

Table 1
Table of Correspondence

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine

SYMBOL		
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those referred to in 37 C.F.R. § § 1.821-1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or

more amino acid residues or to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224).

Such substitutions are preferably made in accordance with those set forth in TABLE 2 as follows:

TABLE 2

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

As used herein, a complementing plasmid describes plasmid vectors that deliver nucleic acids into a packaging cell line for stable integration into a chromosome in the cellular genome.

As used herein, a delivery plasmid is a plasmid vector that carries or delivers nucleic acids encoding a therapeutic gene or gene that encodes a

therapeutic product or a precursor thereof or a regulatory gene or other factor that results in a therapeutic effect when delivered *in vivo* in or into a cell line, such as, but not limited to a packaging cell line, to propagate therapeutic viral vectors.

As used herein, a variety of vectors with different requirements are described. For example, one vector is used to deliver particular nucleic acid molecules into a packaging cell line for stable integration into a chromosome. These types of vectors are generally identified herein as complementing plasmids. A further type of vector described herein carries or delivers nucleic acid molecules in or into a cell line (*e.g.*, a packaging cell line) for the purpose of propagating therapeutic viral vectors; hence, these vectors are generally referred to herein as delivery plasmids. A third "type" of vector described herein is used to carry nucleic acid molecules encoding therapeutic proteins or polypeptides or regulatory proteins or are regulatory sequences to specific cells or cell types in a subject in need of treatment; these vectors are generally identified herein as therapeutic viral vectors or recombinant adenoviral vectors or viral Ad-derived . vectors and are in the form of a virus particle encapsulating a viral nucleic acid containing an expression cassette for expressing the therapeutic gene.

As used herein, a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a therapeutic polypeptide. By the term "substantially homologous" is meant having at least 80%, preferably at least 90%, most preferably at least 95% homology therewith or a lesser percentage of homology or identity and conserved biological activity or function.

The terms "homology" and "identity" are often used interchangeably. In this regard, percent homology or identity may be determined, for example, by comparing sequence information using a GAP computer program. The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default

parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745 (1986), as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*:2444 (1988). Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity.

In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. (See, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotides or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988)). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988). Methods to determine identity and similarity are codified in computer programs. Preferred

computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al., Nucleic Acids Research 12(l)*:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J Molec Biol 215*:403 (1990)).

Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide. As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the heterologous DNA is expressed and a therapeutic product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy may also be used to deliver nucleic acid encoding a gene product to replace a defective gene or supplement a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid may encode a therapeutic compound, such as a growth

factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof.

As used herein, heterologous DNA is DNA that encodes RNA and proteins that are not normally produced *in vivo* by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

Hence, herein heterologous DNA or foreign DNA, refers to a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in the corresponding wild-type adenovirus. It may also refer to a DNA molecule from another organism or species (*i.e.*, exogenous) or from another Ad serotype.

As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures said disease.

Typically, DNA encoding the desired heterologous DNA is cloned into a plasmid vector and introduced by routine methods, such as calcium-phosphate mediated DNA uptake (see, (1981) Somat. Cell. Mol. Genet. 7:603-616) or

microinjection, into producer cells, such as packaging cells. After amplification in producer cells, the vectors that contain the heterologous DNA are introduced into selected target cells.

As used herein, an expression or delivery vector refers to any plasmid or virus into which a foreign or heterologous DNA may be inserted for expression in a suitable host cell — *i.e.*, the protein or polypeptide encoded by the DNA is synthesized in the host cell's system. Vectors capable of directing the expression of DNA segments (genes) encoding one or more proteins are referred to herein as "expression vectors." Also included are vectors that allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

As used herein, a gene is a nucleic acid molecule whose nucleotide sequence encodes RNA or polypeptide. A gene can be either RNA or DNA. Genes may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

As used herein, tropism with reference to an adenovirus refers to the selective infectivity or binding that is conferred on the particle by the fiber protein, such as by the C-terminus portion that comprises the knob.

As used herein, isolated with reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid were obtained. It may also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compound can be substantially purified by the one-step method described in

Smith and Johnson, *Gene 67:*31-40 (1988). The terms isolated and purified are sometimes used interchangeably.

Thus, by "isolated" is meant that the nucleic acid is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

Isolated or purified as it refers to preparations made from biological cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

A preparation of DNA or protein that is "substantially pure" or "isolated" should be understood to mean a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95% of the DNA or protein of interest.

A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of interest. The term "cell extract" is intended to include culture media, especially spent culture media from which the cells have been removed.

As used herein, a packaging cell line is a cell line that provides a missing gene product or its equivalent.

As used herein, an adenovirus viral particle is the minimal structural or functional unit of a virus. A virus can refer to a single particle, a stock of

particles or a viral genome. The adenovirus (Ad) particle is relatively complex and may be resolved into various substructures.

As used herein, "penton" or "penton complex" are preferentially used herein to designate a complex of penton base and fiber. The term "penton" may also be used to indicate penton base, as well as penton complex. The meaning of the term "penton" alone should be clear from the context within which it is used.

As used herein, a plasmid refers to an autonomous self-replicating extrachromosomal circular nucleic acid molecule, typically DNA.

As used herein, a post-transcription regulatory element (PRE) is a regulatory element found in viral or cellular messenger RNA that is not spliced, i.e. intronless messages. Examples include, but are not limited to, human hepatitis virus, woodchuck hepatitis virus, the TK gene and mouse histone gene. The PRE may be placed before a polyA sequence and after a heterologous DNA sequence.

As used herein, pseudotyping describes the production of adenoviral vectors having modified capsid protein or capsid proteins from a different serotype than the serotype of the vector itself. One example, is the production of an adenovirus 5 vector particle containing an Ad37 fiber protein. This may be accomplished by producing the adenoviral vector in packaging cell lines expressing different fiber proteins.

As used herein, promoters of interest herein may be inducible or constitutive. Inducible promoters will initiate transcription only in the presence of an additional molecule; constitutive promoters do not require the presence of any additional molecule to regulate gene expression. a regulatable or inducible promoter may also be described as a promoter where the rate or extent of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include, but are not limited to various compounds or compositions, light, heat, stress and chemical energy sources. Inducible, suppressible and repressible promoters are considered regulatable promoters. Preferred promoters herein, are promoters that are selectively expressed in ocular cells, particularly photoreceptor cells.

As used herein, receptor refers to a biologically active molecule that specifically or selectively binds to (or with) other molecules. The term "receptor protein" may be used to more specifically indicate the proteinaceous nature of a specific receptor.

As used herein, recombinant refers to any progeny formed as the result of genetic engineering. This may also be used to describe a virus formed by recombination of plasmids in a packaging cell.

As used herein, a transgene or therapeutic nucleic acid molecule includes DNA and RNA molecules encoding an RNA or polypeptide. Such molecules may be "native" or naturally-derived sequences; they may also be "non-native" or "foreign" that are naturally- or recombinantly-derived. The term "transgene," which may be used interchangeably herein with the term "therapeutic nucleic acid molecule," is often used to describe a heterologous or foreign (exogenous) gene that is carried by a viral vector and transduced into a host cell.

Therefore, therapeutic nucleotide nucleic acid molecules include antisense sequences or nucleotide sequences which may be transcribed into antisense sequences. Therapeutic nucleotide sequences (or transgenes) all include nucleic acid molecules that function to produce a desired effect in the cell or cell nucleus into which said therapeutic sequences are delivered. For example, a therapeutic nucleic acid molecule can include a sequence of nucleotides that encodes a functional protein intended for delivery into a cell which is unable to produce that functional protein.

As used herein, the vitreous of the eye refers to material that fills the chamber behind the lens of the eye (i.e., vitreous humor or vitreous body).

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of the DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be *cis* acting or

may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

Thus, promoters are nucleic acid fragments that contain a DNA sequence that controls the expression of a gene located 3' or downstream of the promoter. The promoter is the DNA sequence to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene, typically located 3' of the promoter. A promoter also includes DNA sequences that direct the initiation of transcription, including those to which RNA polymerase specifically binds. If more than one nucleic acid sequence encoding a particular polypeptide or protein is included in a therapeutic viral vector or nucleotide sequence, more than one promoter or enhancer element may be included, particularly if that would enhance efficiency of expression.

A regulatable or inducible promoter may be described as a promoter wherein the rate of RNA polymerase binding and initiation is modulated by external stimuli. (see, *e.g.*, U.S. Patent Nos. 5,750,396 and 5,998,205). Such stimuli include various compounds or compositions, light, heat, stress, chemical energy sources, and the like. Inducible, suppressible and repressible promoters are considered regulatable promoters.

Regulatable promoters may also include tissue-specific promoters. Tissue-specific promoters direct the expression of the gene to which they are operably linked to a specific cell type. Tissue-specific promoters cause the gene located 3' of it to be expressed predominantly, if not exclusively, in the specific cells where the promoter expressed its endogenous gene. Typically, it appears that if a tissue-specific promoter expresses the gene located 3' of it at all, then it is expressed appropriately in the correct cell types (see, *e.g.*, Palmiter et al. (1986) Ann. Rev. Genet. 20: 465-499).

As used herein, the phrase "operatively linked" generally means the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form, whereby control sequences on one segment control expression or replication or other such control of other segments. The two segments are not necessarily contiguous.

As used herein, exogenous encompasses any therapeutic composition that is administered by the therapeutic methods provided herein. Thus, exogenous may also be referred to herein as foreign, or non-native or other equivalent expression.

B. Ad37 fiber tropism

The adenovirus fiber protein is a major determinant of adenovirus tropism (Gall *et al.* (1996) *J. Virol. 70*:2116-2123; Stevenson *et al.* (1995) *J. Virol. 69*:2850-2857). The fiber protein extends from the capsid and mediates viral binding to the cell surface by binding to specific cell receptors (Philipson *et al.* (1968) *J. Virol. 2*:1064-1075). The fiber is a trimeric protein that includes an N-terminal tail domain that interacts with the adenovirus penton base, a central shaft domain of varying length, and a C-terminal knob domain that contains the cell receptor binding site (Chroboczek *et al.* (1995) *Curr.Top.Microbiol.Immunol. 199*:163-200; Riurok *et al.* (1990) *J.Mol.Biol. 215*:589-596; Stevenson *et al.* (1995) *J. Virol. 69*:2850-2857). Fiber proteins of most adenovirus subgroups have been shown to bind specifically or selectively to the 46 kDa coxsackievirus-adenovirus receptor (CAR), (Bergelson *et al.* (1997) *Science 275*:1320-1323; Roelvink *et al.* (1998) *J. Virol. 72*:7909-7915). CAR appears to be expressed in a variety of human tissues, including the lung, at various levels (Bergelson *et al.* (1997) *Science 275*:1320-1323), but Ad37 binds poorly to lung epithelial cells (Huang *et al.* (1999) *J. Virol. 73*:2798-2802). This suggests that the tropism of this serotype may be influenced by factors independent of CAR expression.

Structural and biochemical data also suggest that distinct receptor binding sites are located on different regions of the Ad5 and Ad37 fiber knobs. Adopting the nomenclature of Xia et al. (Xia *et al.* (1994) *Structure 2*:1259-1270), the receptor binding site for Ad5 is located at the AB-loop on the side of the fiber knob (Bewley *et al.* (1999) *Science 286*:1579-1583; Roelvink *et al.* (1999) *Science 286*:1568-1571). It is known that a lysine residue at position 240 of the Ad37 fiber, located in the CD-loop, is important for receptor binding (Huang *et al.* (1999) *J. Virol. 73*:2798-2802). The co-crystal structure of the Ad12 knob and the N-terminal domain of CAR (Bewley *et al.* (1999) *Science 286*:1579-1583) show that the CD-loop does not contact CAR. It thus appears

that different regions of the Ad5 and Ad37 fiber knobs recognize distinct cell receptors.

A 46 kDa receptor for coxsackieviruses and adenoviruses (CAR) mediates attachment for many adenovirus serotypes. The wide distribution of CAR fails to explain why certain adenovirus serotypes (i.e. Ad37) are highly associated with severe ocular infections such as epidemic keratoconjunctivitis (EKC). Ad37 does not use CAR, but instead uses a glycoprotein that contains sialic acid as its primary receptor (Arnberg *et al.* ((2000) *J. Virol. 74*:42-48). The modest number of Ad37 binding sites per cell (Huang *et al.* (1999) *J. Virol. 73*:2798-2802) also suggests that Ad37 recognizes a specific glycoprotein as its primary receptor for binding to conjunctival cells.

Adenovirus type 37 (subgroup D) has been associated with infections of the eye and genital tract. The tropism of Ad37 derives from the binding preference of its fiber protein, which binds to a receptor located on the surface of cells including Chang C, conjunctival epithelial cell line (Huang *et al.* (1999) *J. Virology* 73:2798-2802).

A protein receptor that is preferentially expressed on conjunctival cells to which Ad37 fiber binds is shown herein. The preferential expression of the Ad37 receptor protein on conjunctival cells suggests that this receptor likely influences Ad37 tropism and should play a key role in ocular pathogenesis. It is shown herein that Ad37 uses a distinct protein receptor that is selectively expressed on conjunctival cells. It is shown that Ad37 binds well to conjunctival cells (Chang C), but poorly to lung carcinoma cells (A549). To determine if infection correlated with cell binding, an Ad5 vector containing the Ad37 fiber protein was constructed. The 'pseudotyped' vector delivered transgenes to Chang C cells better than to A549 cells. Ad37 binding was abolished by protease treatment of Chang C cells, indicating the receptor is a membrane protein. Ad37 binding to conjunctival cells is shown herein to be calcium-dependent. It is also shown that Ad37 infection was not inhibited by a function-blocking anti-CAR monoclonal antibody, which is a feature distinct from Ad5 fiber interaction with CAR. Using a virus overlay protein blot assay (VOPBA), calcium-dependent Ad37 binding to a 50 KDa membrane protein on Chang C

cells, but not A549 cells was detected. Ad19p a closely related serotype that fails to bind to conjunctival cells, does not recognize the 50 kDa protein. Together, these data indicate that the 50 kDa protein is a candidate receptor for Ad37 on conjunctival cells.

Significantly, it is also shown herein that, upon administration of the vector to the vitreous humor, the recombinant adenovirus with the Ad37 fiber preferentially and selectively binds to photoreceptor cells. Hence, a recombinant adenoviral delivery vehicle that has an Ad37 fiber protein can serve as a vector for delivery of therapeutic agents to the eye for treatment of ocular disorders, including genetic and acquired disorders. The identification of the receptor for Ad37 and the resulting recognition of Ad37 tropism allows targeting of adenovirus vectors to specific human ocular cells.

As noted, fiber plays a crucial role in adenovirus infection by attaching the virus to a specific receptor on a cell surface. Hexon, penton and fiber capsomeres are the major components on the surface of the virion. The fiber is an elongated protein which exists as a trimer of three identical polypeptides (polypeptide IV) of 582 amino acids in length. An adenovirus fiber includes three domains: an N-terminal tail domain that interacts with penton base; a shaft composed of variable numbers of repeats of a 15-amino-acid segment that forms beta-sheet and beta-bends; and a knob at the C-terminus ("head domain") that contains the type-specific antigen and is responsible for binding to the cell surface receptor. The gene encoding the fiber protein from Ad2 has been expressed in human cells and has been shown to be correctly assembled into trimers, glycosylated and transported to the nucleus (see, *e.g.*, Hong and Engler, *Virology 185*: 758-761, 1991). Thus, alteration of the fiber in recombinant Ad vectors can lead to alteration in gene delivery.

As shown herein, alteration of fiber in recombinant Ad vectors such that the fiber is derived from Ad37 or another adenovirus serotype D, provides a means for selective delivery of a recombinant virus to particular cells in the eye, including conjunctival cells, and most significantly photoreceptors, thereby providing a means for targeted delivery to photoreceptor cells.

Photoreceptor cells are implicated in a number of hereditary and acquired retinal degenerative disorders. In addition, photoreceptor cells are located such that products produced therein can be delivered to other areas of the eye by virtue of the blood flow in the vicinity of the photoreceptor cells and also by virtue of the proximity of the photoreceptors to the retinal pigmented epithelium (RPE) and other retinal cells.

Hence it is contemplated herein that the recombinant viral vector will include a packaged recombinant adenovirus genome containing at least the minimal elements for replication and packaging; heterologous DNA encoding a desired gene product, typically a therapeutic product or plurality of products, such as several trophic factors, whose combined activity is effective for treating a disorder, such as a retinal degenerative disorder; and the resulting virion particles will include a fiber that has a sufficient portion to confer specific targeting to photoreceptor cells when the recombinant viral particles are introduced into the aqueous humor of a mammalian, preferably a human, eye, or otherwise contacted with the photoreceptor cells. The fiber may be a chimeric protein that has been modified for effective interaction with other coat structural proteins, such as penton. In addition, the fiber may be modified to include other elements that alter its tropism to permit binding to other cells as well (see, *e.g.*, U.S. Patent Nos. 5,756,086 and 5,543,328, International PCT application No. WO 95/26412 and WO 98/44121 and Krasnykh, *et al.* (*J. Virol. 70:* 6839-46, 1996).

C. Construction of the viral particles

1. Selection of viral genome and fiber protein

Methods for preparing recombinant adenoviral vectors for gene product delivery are well known. Preferred among those are the methods exemplified herein (see EXAMPLES) and also described in copending U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265, filed January 14, 2000, which claims priority to U.S. provisional application Serial No. 60/115,920, as does U.S. application Serial No. 09/482,682)).

As noted, any desired recombinant adenovirus is contemplated for use in the methods herein as long as the viral genome is packaged in a capsid that includes at least the portion of a fiber protein that provides selective binding to photoreceptor cells. This fiber protein is preferably from an adenovirus type D serotype and is preferably an Ad37 fiber. The fiber protein should retain the knob region at the C-terminus ("head domain") from the Ad virus of subgroup D that contains the type-specific antigen and is responsible for binding to the cell surface receptor. Hence the fiber protein can be a chimeric fiber protein as long as it retains a sufficient portion of the type D serotype to specifically or selectively bind to photoreceptor cells. Generally the portion retained will be all or a portion of the knob region. The precise amount of knob region required can be determined empirically by including portions thereof and identifying the minimum residues from and Ad type D serotype, preferably Ad37, to effect selective targeting of a virion packaged with such fiber to photoreceptors in the eye upon introduction of the packaged virion into the aqueous humor.

Recombinant adenovirus containing heterologous nucleic acids that encode a desired product, such a gene to correct a genetic defect, may be made by any methods known to those of skill in the art. The viruses must be packaged in a cell line that results in expression of fiber on the particles that specifically, electively or preferentially targets (binds and results in internalization) the viral particle to cells in the eye. The fiber protein from Ad37 and other Adenoviruses of serotype D that infect the eye effects such targeting. The resulting adenovirus particles that express such fiber is administered by intraocular injection, subretinal injection, particularly intravitreal injection, or any means that results in preferential accumulation in photoreceptor cells.

The family of Adenoviridae includes many members with at least 47 known serotypes of human adenovirus (Ad1-Ad47) (Shenk, *Virology*, Chapter 67, *in* Fields *et al.*, eds. Lippincott-Raven, Philadelphia, 1996,) as well as members of the genus Mastadenovirus including human, simian, bovine, equine, porcine, ovine, canine and opossum viruses and members of the Aviadenovirus genus, including bird viruses, such as CELO.

Thus it is contemplated that the methods herein can be applied to any recombinant viral vectors derived from any adenovirus species. One of skill in the art would have knowledge of the different adenoviruses (see, *e.g.*,Shenk, *Virology*, Chapter 67, *in* Fields *et al.,* eds. Lippincott-Raven, Philadelphia, 1996,) and can construct recombinant viruses containing portions of the genome of any such virus.

In the exemplified embodiment, viral particles with Ad37 fiber were prepared. Site-directed mutations were made to the Ad37 fiber gene to make the tail sequence more closely match that of Ad5 to facilitate Ad37 fiber binding to the Ad5 penton base. The plasmid for the expression of the Ad37 fiber protein, pDV80, contains the CMV promoter, the adenovirus type 5 tripartite leader (TPL), and the modified Ad37 fiber gene sequence. Genes of interest, such as nucleic acid encoding the β subunit of cGMP phosphodiesterase (βPDE), β-glucuronidase, rhodopsin, growth factors, anti-cancer agents, growth factor receptors and other anti-angiogenic agents, and anti-apoptotic agents, can be incorporated into these vectors using the methods known to those of skill in the art and exemplified herein.

Known adenovirus vectors, previously constructed for intraocular therapy (see, *e.g.*, Bennett *et al.* (1996) *Nature Medicine* *2*:649-654, which provides an Ad virus encoding βPDE for treatment of retinitis pigmentosa; Cayouette *et al.* (1998) *Human Gene Therapy* *8*:423-430, which provides an Ad vector that expresses CNTF for treatment of retinitis pigmentosa and other retinal degenerative diseases; and Li *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* *92*:7700-7704, which provides an Ad virus vector that encodes a human β-glucuronidase for treatment of lysosomal storage disease caused by β-glucuronidase deficiency) can be modified by repackaging the recombinant genome using a packaging line that expresses an Ad37 fiber or other D serotype fiber.

For exemplification, nucleic acid encoding GFP was incorporated into these vectors as a means to visualize their localization. Other genes, such as genes that encode therapeutic products, my be included in place of or in addition to GFP.

Plasmid pDV80 was electroporated into E1-2a S8 cells and stable lines were selected. The fiber-deleted vectors Ad5.βgal.ΔF and Ad5.GFP.ΔF were grown in cells in a resulting cell line, designated 705, to produce virions, which express the Ad37 fiber (Ad5.βgal.ΔF/37F and Ad5.GFP.ΔF/37F) and CsCl-purified. These virions selectively transduce photoreceptor cells when injected intraocularly into the vitreous humor.

2. **Packaging**

Recombinant adenoviral vectors generally have at least a deletion in the first viral early gene region, referred to as E1, which includes the E1a and E1b regions. Deletion of the viral E1 region renders the recombinant adenovirus defective for replication and incapable of producing infectious viral particles in subsequently-infected target cells. Thus, to generate E1-deleted adenovirus genome replication and to produce virus particles requires a system of complementation which provides the missing E1 gene product. E1 complementation is typically provided by a cell line expressing E1, such as the human embryonic kidney packaging cell line, i.e. an epithelial cell line, called 293. Cell line 293 contains the E1 region of adenovirus, which provides E1 gene region products to "support" the growth of E1-deleted virus in the cell line (see, *e.g.*, Graham *et al., J. Gen. Virol. 36*: 59-71, 1977). Additionally, cell lines that may be usable for production of defective adenovirus having a portion of the adenovirus E4 region have been reported (WO 96/22378).

Multiply deficient adenoviral vectors and complementing cell lines have also been described (WO 95/34671, U.S. Patent No. 5,994,106).

Copending U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265, filed January 14, 2000)) provides packaging cell lines that support viral vectors with deletions of major portions of the viral genome, without the need for helper viruses and also provides cell lines and helper viruses for use with helper-dependent vectors. The packaging cell line has heterologous DNA stably integrated into the chromosomes of the cellular genome. The heterologous DNA sequence encodes one or more adenovirus regulatory and/or structural polypeptides that complement the genes deleted or mutated in the adenovirus vector genome to

be replicated and packaged. The packaging cell line express, for example, one or more adenovirus structural proteins, polypeptides, or fragments thereof, such as penton base, hexon, fiber, polypeptide IIIa, polypeptide V, polypeptide VI, polypeptide VII, polypeptide VIII, and biologically active fragments thereof. The expression can be constitutive or under the control of a regulatable promoter. These cell lines are designed for expression of recombinant adenoviruses intended for delivery of therapeutic products.

Particular packaging cell lines complement viral vectors having a deletion or mutation of a DNA sequence encoding an adenovirus structural protein, regulatory polypeptides E1A and E1B, and/or one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof.

The packaging cell lines are produced by introducing each DNA molecule into the cells and then into the genome via a separate complementing plasmid or plurality of DNA molecules encoding the complementing proteins can be introduced via a single complementing plasmid. Of interest herein, is a variation in which the complementing plasmid includes DNA encoding adenovirus fiber protein (or a chimeric or modified variant thereof), from Ad virus of subgroup D, such as Ad 37, polypeptide or fragment thereof.

For therapeutic applications, the delivery plasmid further includes a nucleotide sequence encoding a foreign polypeptide. Exemplary delivery plasmids include, but are not limited to, pDV44, pΔE1Bβ-gal and pΔE1sp1B. In a similar or analogous manner, therapeutic genes may be introduced.

The cell further includes a complementing plasmid encoding a fiber as contemplated herein; the plasmid or portion thereof is integrated into a chromosome(s) of the cellular genome of the cell.

In one embodiment, a composition comprises a cell containing first and second delivery plasmids wherein a first delivery plasmid comprises an adenovirus genome lacking a nucleotide sequence encoding fiber and incapable of directing the packaging of new viral particles in the absence of a second delivery plasmid, and a second delivery plasmid comprises an adenoviral genome capable of directing the packaging of new viral particles in the presence of the first delivery plasmid.

In a variation, the packaging cell line expresses fiber protein or chimeric variant thereof from an Ad virus of subgroup D, preferably Ad37, serotype or it can be any fiber protein but one that has been modified to include the portion of the Ad virus of subgroup D, such as Ad37, responsible for selective targeting to photoreceptors upon introduction into the vitreous humor of the eye of a mammal, preferably a human. The fiber protein can be further modified to include a non-native amino acid residue sequence that targets additional specific receptors. In all instances, the modification should not disrupt trimer formation or transport of fiber into the nucleus. In another variation, the non-native amino acid residue sequence alters the binding specificity of the fiber for a targeted cell type. The structural protein is fiber can include amino acid residue sequences from more than one adenovirus serotype. The nucleotide sequences encoding fiber protein or polypeptide need not be modified solely at one or both termini; fiber protein, may be modified "internally" as well as at the termini.

Additional nucleic acid fragments can encode polypeptides that are added to the fiber protein. In one variation, the non-native amino acid residue sequence is coupled to the carboxyl terminus of the fiber. In another, the non-native amino acid residue sequence further includes a linker sequence. Alternatively, the fiber protein further comprises a ligand coupled to the linker. Suitable ligands include, but are not limited to, ligands that specifically or selectively bind to a cell surface receptor and ligands that can be used to couple other proteins or nucleic acid molecules. Typically, the packaging cell lines will contain nucleic acid encoding the fiber protein or modified protein stably integrated into a chromosome or chromosomes in the cellular genome.

The packaging cell line can be derived from a procaryotic cell line or from a eukaryotic cell line. While various embodiments suggest the use of mammalian cells, and more particularly, epithelial cell lines, a variety of other, non-epithelial cell lines are used in various embodiments. Thus, while various embodiments disclose the use of a cell line selected from among the 293, A549, W162, HeLa, Vero, 211, and 211A cell lines, and any other cell lines suitable for such use are likewise contemplated herein.

3. Components of the nucleic acid molecule included in the particle

A recombinant viral vector or therapeutic viral vector for use in the methods herein, typically includes a nucleic acid fragment that encodes a protein or polypeptide molecule, or a biologically active fragment thereof, or other regulatory sequence, that is intended for use in therapeutic applications.

The nucleic acid molecule to be packaged in the viral particle also may include an enhancer element and/or a promoter located 3' or 5' to and controlling the expression of the therapeutic product-encoding nucleic acid molecule if the product is a protein. Further, for purposes herein, the promoter and/or other transcriptional and translational regulatory sequences controlling expression of the product is preferably one that is expressed specifically in the targeted cells, such as the a photoreceptor-specific promoter, such as a rhodopsin gene promoter.

The nucleic acid molecule to be packaged in viral capsid includes at least 2 different operatively linked DNA segments. The DNA can be manipulated and amplified by PCR as described herein and by using standard techniques, such as those described in *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Sambrook *et al.*, eds., Cold Spring Harbor, New York (1989). Typically, to produce such molecule, the sequence encoding the selected polypeptide and the promoter or enhancer are operatively linked to a DNA molecule capable of autonomous replication in a cell either *in vivo* or *in vitro*. By operatively linking the enhancer element or promoter and nucleic acid molecule to the vector, the attached segments are replicated along with the vector sequences.

Thus, the recombinant DNA molecule (rDNA) is a hybrid DNA molecule comprising at least 2 nucleotide sequences not normally found together in nature. In various preferred embodiments, one of the sequences is a sequence encoding an Ad-derived polypeptide, protein, or fragment thereof. The nucleic acid molecule intended to be packaged is from about 20 base pairs to about 40,000 base pairs in length, preferably about 50 bp to about 38,000 bp in length. In various embodiments, the nucleic acid molecule is of sufficient length to encode one or more adenovirus proteins or functional polypeptide portions thereof. Since individual Ad polypeptides vary in length from about 19 amino acid residues to about 967 amino acid residues, encoding nucleic acid molecules

from about 50 bp up to about 3000 bp, depending on the number and size of individual polypeptide-encoding sequences that are "replaced" in the viral vectors by therapeutic product-encoding nucleic acid molecules.

Preferably the molecule includes an adenovirus tripartite leader (TPL) nucleic acid sequence operatively linked to an intron containing RNA processing signals (such as for example, splice donor or splice acceptor sites) suitable for expression in the packaging cell line. Most preferably the intron contains a splice donor site and a splice acceptor site. Alternatively, the TPL nucleotide sequence may not comprise an intron. The intron includes any sequence of nucleotides that function in the packaging cell line to provide RNA processing signals, including splicing signals. Introns have been well characterized from a large number of structural genes, and include but are not limited to a native intron 1 from adenovirus, such as Ad5's TPL intron 1; others include the SV40 VP intron; the rabbit beta-globin intron, and synthetic intron constructs (see, *e.g.*, Petitclerc *et al.* (1995)*J. Biothechnol.*, *40*:169; and Choi *et al.* (19910 *Mol. Cell. Biol.*, *11*:3070).

The nucleic acid molecule encoding the TPL includes either (a) first and second TPL exons or (b) first, second and third TPL exons, where each TPL exon in the sequence is selected from among the complete TPL exon 1, partial TPL exon 1, complete TPL exon 2 and complete TPL exon 3. A complete exon is one which contains the complete nucleic acid sequence based on the sequence found in the wild type viral genome. Preferably the TPL exons are from Ad2, Ad3, Ad5, Ad7 and the like, however, they may come from any Ad serotype, as described herein. A preferred partial TPL exon 1 is described in the Examples. The use of a TPL with a partial exon 1 has been reported (International PCT application No. WO 98/13499).

The intron and the TPL exons can be operatively linked in a variety of configurations to provide a functional TPL nucleotide sequence. An intron may not be a part of the construct. For example, the intron can be positioned between any of TPL exons 1, 2 or 3, and the exons can be in any order of first and second, or first/second/third. The intron can also be placed preceding the first TPL exon or following the last TPL exon. In a preferred embodiment,

complete TPL exon 1 is operatively linked to complete TPL exon 2 operatively linked to complete TPL exon 3. In a preferred variation, adenovirus TPL intron 1 is positioned between complete TPL exon 1 and complete TPL exon 2. It may also be possible to use analogous translational regulators from other viral systems such as rabiesvirus.

A preferred "complete" TPL nucleic acid molecule containing complete TPL exons 1, 2 and 3 with adenovirus intron 1 inserted between exons 1 and 2 has a nucleotide sequence shown in SEQ ID NO: 32. A preferred "partial" TPL nucleic acid molecule containing partial TPL exon 1 and complete TPL exons 2 and 3 in that order has a nucleotide sequence shown in SEQ ID NO: 26. The construction of these preferred TPL nucleotide sequences is described in the Examples.

Thus, preferred expression cassettes and complementing plasmids for expressing adenovirus structural genes, particularly fiber protein, contain an adenovirus TPL nucleotide sequence as described herein.

4. Complementing Plasmids

Also contemplated are the use of nucleic acid molecules, typically in the form of DNA plasmid vectors, which are capable of expression of an adenovirus structural protein or regulatory protein. Because these expression plasmids are used to complement the defective genes of a recombinant adenovirus vector genome, the plasmids are referred to as complementing or complementation plasmids.

The complementing plasmid contains an expression cassette, a nucleotide sequence capable of expressing a protein product encoded by the nucleic acid molecule. Expression cassettes typically contain a promoter and a structural gene operatively linked to the promoter. The complementing plasmid can further include a sequence of nucleotides encoding TPL nucleotide to enhance expression of the structural gene product when used in the context of adenovirus genome replication and packaging.

A complementing plasmid can include a promoter operatively linked to a sequence of nucleotides encoding an adenovirus structural polypeptide, such as, but are not limited to, penton base; hexon; fiber; polypeptide IIIa; polypeptide V;

polypeptide VI; polypeptide VII; polypeptide VIII; and biologically active fragments thereof. In another variation, a complementing plasmid may also include a sequence of nucleotides encoding a first adenovirus regulatory polypeptide, a second regulatory polypeptide, and/or a third regulatory polypeptide, and any combination of the foregoing.

Plasmid pDV80 is a preferred plasmid herein. Other plasmids constructed in an analogous manner to encode modified fiber proteins and chimeric fiber proteins are also contemplated herein.

5. Nucleic Acid Molecule Synthesis

A nucleic acid molecule comprising synthetic oligonucleotides can be prepared using any suitable method, such as the phosphotriester or phosphodiester methods (see, *e.g.*, Narang (1979) *et al., Meth. Enzymol.,* 68:90; U.S. Patent No. 4,356,270; and Brown *et al.,* (1979) *Meth. Enzymol.,* 68:109). For oligonucleotides, the synthesis of the family members can be conducted simultaneously in a single reaction vessel, or can be synthesized independently and later admixed in preselected molar ratios. For simultaneous synthesis, the nucleotide residues that are conserved at preselected positions of the sequence of the family member can be introduced in a chemical synthesis protocol simultaneously to the variants by the addition of a single preselected nucleotide precursor to the solid phase oligonucleotide reaction admixture when that position number of the oligonucleotide is being chemically added to the growing oligonucleotide polymer. The addition of nucleotide residues to those positions in the sequence that vary can be introduced simultaneously by the addition of amounts, preferably equimolar amounts, of multiple preselected nucleotide precursors to the solid phase oligonucleotide reaction admixture during chemical synthesis. For example, where all four possible natural nucleotides (A,T,G and C) are to be added at a preselected position, their precursors are added to the oligonucleotide synthesis reaction at that step to simultaneously form four variants (see, *e.g.*, Ausubel *et al. (Current Protocols in Molecular Biology,* Suppl. 8. p.2.11.7, John Wiley & Sons, Inc., New York ,1991).

Nucleotide bases other than the common four nucleotides (A,T,G or C), or the RNA equivalent nucleotide uracil (U), can also be used. For example, it is well known that inosine (I) is capable of hybridizing with A, T and G, but not C. Examples of other useful nucleotide analogs are known in the art and may be found referred to in 37 C.F.R. §1.822.

Thus, where all four common nucleotides are to occupy a single position of a family of oligonucleotides, that is, where the preselected nucleotide sequence is designed to contain oligonucleotides that can hybridize to four sequences that vary at one position, several different oligonucleotide structures are contemplated. The composition can contain four members, where a preselected position contains A,T,G or C. Alternatively, a composition can contain two nucleotide sequence members, where a preselected position contains I or C, and has the capacity to hybridize at that position to all four possible common nucleotides. Finally, other nucleotides may be included at the preselected position that have the capacity to hybridize in a non-destabilizing manner with more than one of the common nucleotides in a manner similar to inosine.

Similarly, larger nucleic acid molecules can be constructed in synthetic oligonucleotide pieces, and assembled by complementary hybridization and ligation, as is well known.

D. Adenovirus Expression Vector Systems

The adenovirus vector genome that is encapsulated in the virus particle and that expresses exogenous genes in a gene therapy setting is a key component of the system. Thus, the components of a recombinant adenovirus vector genome include the ability to express selected adenovirus structural genes, to express a desired exogenous protein, and to contain sufficient replication and packaging signals that the genome is packaged into a gene delivery vector particle. The preferred replication signal is an adenovirus inverted terminal repeat containing an adenovirus origin of replication, as is well known and described herein.

Although adenovirus include many proteins, not all adenovirus proteins are required for assembly of a recombinant adenovirus particle (vector). Thus, deletion of the appropriate genes from a recombinant Ad vector permits accommodation of even larger "foreign" DNA segments.

A preferred recombinant adenovirus vector genome is "helper independent" so that genome can replicate and be packaged without the help of a second, complementing helper virus. Complementation is provided by a packaging cell.

In a preferred embodiment, the adenovirus vector genome does not encode a functional adenovirus fiber protein. A non-functional fiber gene refers to a deletion, mutation or other modification to the adenovirus fiber gene such that the gene does not express any or insufficient adenovirus fiber protein to package a fiber-containing adenovirus particle without complementation of the fiber gene by a complementing plasmid or packaging cell line. Such a genome is referred to as a "fiberless" genome, not to be confused with a fiberless particle. Alternatively, a fiber protein may be encoded but is insufficiently expressed to result in a fiber containing particle.

Thus, contemplated for use are helper-independent fiberless recombinant adenovirus vector genomes that include genes that (a) express all adenovirus structural gene products but express insufficient adenovirus fiber protein to package a fiber-containing adenovirus particle without complementation of said fiber gene, (b) express an exogenous protein, and (c) contain an adenovirus packaging signal and inverted terminal repeats containing adenovirus origin of replication.

The adenovirus vector genome is propagated in the laboratory in the form of rDNA plasmids containing the genome, and upon introduction into an appropriate host, the viral genetic elements provide for viral genome replication and packaging rather than plasmid-based propagation. Exemplary methods for preparing an Ad-vector genome are described in the Examples.

A vector herein includes a nucleic acid (preferably DNA) molecule capable of autonomous replication in a cell and to which a DNA segment, *e.g.*, a gene or polynucleotide, can be operatively linked to bring about replication of the

attached segment. For purposes herein, one of the nucleotide segments to be operatively linked to vector sequences encodes at least a portion of a therapeutic nucleic acid molecule. As noted above, therapeutic nucleic acid molecules include those encoding proteins and also those that encode regulatory factors that can lead to expression or inhibition or alteration of expression of a gene product in a targeted cell.

1. **Nucleic Acid Gene Expression Cassettes**

In various embodiments, a peptide-coding sequence of the therapeutic gene is inserted into an expression vector and expressed; however, it is also feasible to construct an expression vector which also includes some non-coding sequences as well. Preferably, however, non-coding sequences are excluded. Alternatively, a nucleotide sequence for a soluble form of a polypeptide may be utilized. Another preferred therapeutic viral vector includes a nucleotide sequence encoding at least a portion of a therapeutic nucleotide sequence operatively linked to the expression vector for expression of the coding sequence in the therapeutic nucleotide sequence.

The choice of viral vector into which a therapeutic nucleic acid molecule is operatively linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, vector replication and protein expression, and the host cell to be transformed – these being limitations inherent in the art of constructing recombinant DNA molecules. Although certain adenovirus serotypes are recited herein in the form of specific examples, it should be understood that the use of *any* adenovirus serotype, including hybrids and derivatives thereof are contemplated.

A translatable nucleotide sequence is a linear series of nucleotides that provide an uninterrupted series of at least 8 codons that encode a polypeptide in one reading frame. Preferably, the nucleotide sequence is a DNA sequence. The vector itself may be of any suitable type, such as a viral vector (RNA or DNA), naked straight-chain or circular DNA, or a vesicle or envelope containing the nucleic acid material and any polypeptides that are to be inserted into the cell.

2. Promoters

As noted elsewhere herein, an expression nucleic acid in an Ad-derived vector may also include a promoter, particularly a tissue or cell specific promoter, preferably one expressed in ocular cells, particularly photoreceptors.

Promoters contemplaged for use herein include regulatable (inducible) as well as constitutive promoters, which may be used, either on separate vectors or on the same vector. Some useful regulatable promoters are those of the CREB-regulated gene family and include inhibin, gonadotropin, cytochrome c, glucagon, and the like. (See, *e.g.*, International PCT application No. WO 96/14061). Preferably the promoter selected is from a photoreceptor-specific gene, such as a rhodopsin gene or gene that encodes a protein that regulates rhodopsin expression.

E. Formulation and administration

Compositions containing therapeutically effective concentrations of recombinant adenovirus delivery vectors are provided. These are for delivery of therapeutic gene products to cells, particularly cells express a particular 50 kDa receptor or other receptor with which the vectors interact. These cells include cells of the eye and genital tract. Of particular interest are photoreceptor cells of the eye. Administration is effected by any means through which contacting with the photoreceptors is effected. Preferable modes of administration include, but are not limited to, subretinal injection, particularly intravitreal injection, to provide access to photoreceptor cells.

The recombinant viral compositions may also be formulated for implantation into the anterior or posterior chamber of the eye, preferably the vitreous cavity, in sustained released formulations, such as those adsorbed to biodegradable supports, including collagen sponges, or in liposomes. Sustained release formulations may be formulated for multiple dosage administration, so that during a selected period of time, such as a month or up to about a year, several dosages are administered. Thus, for example, liposomes may be prepared such that a total of about two to up to about five or more times the single dosage is administered in one injection.

The vectors are formulated in an ophthalmologically acceptable carrier for intraocular, preferably intravitreal, administration in a volume of between about 0.05 ml and 0.150 ml, preferably about 0.05 and 0.100 ml.

The composition can be provided in a sealed sterile vial containing an amount of a compound of formula I, that upon intraocular administration will deliver a sufficient amount of viral particles to the photoreceptors in a volume of about 50 to 150 μl, containing at least about 10^7, more preferably at least about 10^8 plaque forming units in such volume. Typically, the vials will, thus, contain about 0.150 ml of the composition.

To prepare compositions the viral particles are dialzyed into a suitable ophthalmologically acceptable carrier or viral particles, for example, may be concentrated and/or mixed therewith. The resulting mixture may be a solution, suspension or emulsion. In addition, the viral particles may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active agents for the particular disorder treated.

For administration by intraocular injection or via eyedrops, suitable carriers include, but are not limited to, physiological saline, phosphate buffered saline (PBS), balanced salt solution (BSS), lactate Ringers solution, and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. Suitable ophthalmologically acceptable carriers are known. Solutions or mixtures intended for ophthalmic use may be formulated as 0.01% - 10% isotonic solutions, pH about 5-7, with appropriate salts [see, e.g., U.S. Patent No. 5,116,868, which describes typical compositions of ophthalmic irrigation solutions and solutions for local application]. Such solutions, which have a pH adjusted to about 7.4, contain, for example, 90-100 mM sodium chloride, 4-6 mM dibasic potassium phosphate, 4-6 mM dibasic sodium phosphate, 8-12 mM sodium citrate, 0.5-1.5 mM magnesium chloride, 1.5-2.5 mM calcium chloride, 15-25 mM sodium acetate, 10-20 mM D.L.-sodium β-hydroxybutyrate and 5-5.5 mM glucose.

The compositions may be prepared with carriers that protect them from rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and other types of implants that may be placed directly into the anterior or posterior chamber or vitreous cavity of the eye. The compositions may also be administered in pellets, such as Elvax pellets (ethylene-vinyl acetate copolymer resin).

Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers. For example, liposome formulations may be prepared by methods known to those of skill in the art [see, e.g., Kimm et al. (1983) Bioch. Bioph. Acta 728:339-398; Assil et al. (1987) Arch Ophthalmol. 105:400; and U.S. Patent No. 4,522,811]. The viral particles may be encapsulated into the aqueous phase of liposome systems.

The active materials can also be mixed with other active materials, that do not impair the desired action, or with materials that supplement the desired action or have other action, including viscoelastic materials, such as hyaluronic acid, which is sold under the trademark HEALON, which is a solution of a high molecular weight (MW) of about 3 millions fraction of sodium hyaluronate [manufactured by Pharmacia, Inc; see, e.g., U.S. Patent Nos. 5,292,362, 5,282,851, 5,273,056, 5,229,127, 4,517,295 and 4,328,803], VISCOAT [fluorine-containing (meth)acrylates, such as, 1H,1H,2H,2H-heptadecafluorodecylmethacrylate; see, e.g., U.S. Patent Nos. 5,278,126, 5,273,751 and 5,214,080; commercially available from Alcon Surgical, Inc.], ORCOLON [see, e.g., U.S. Patent No. 5,273,056; commercially available from Optical Radiation Corporation], methylcellulose, methyl hyaluronate, polyacrylamide and polymethacrylamide [see, e.g., U.S. Patent No. 5,273,751]. The viscoelastic materials are present generally in amounts ranging from about 0.5 to 5.0%, preferably 1 to 3% by weight of the conjugate material and serve to coat and protect the treated tissues. The compositions may also include a dye, such as

methylene blue or other inert dye, so that the composition can be seen when injected into the eye. Additional active agents may be included.

The compositions can be enclosed in ampules, disposable syringes or multiple or single dose vials made of glass, plastic or other suitable material. Such enclosed compositions can be provided in kits. In particular, kits containing vials, ampules or other containers, preferably disposable vials with sufficient amount of the composition to deliver about 0.100 ml thereof, and disposable needles, preferably self sealing 25-30 gauge needles, are provided herein.

Finally, the compounds may be packaged as articles of manufacture containing packaging material, typically a vial, an ophthalmologically acceptable composition containing the viral particles and a label that indicates the therapeutic use of the composition.

Also provided are kits for practice of the methods herein. The kits contain one or more containers, such as sealed vials, with sufficient composition for single dosage administration, and one or more needles, such as self sealing 25-33 gauge needles, preferably 33 gauge or smaller needles, precisely calibrated syringes or other precisely calibrated delivery device, suitable for intravitreal injection.

Administration of the composition is preferably by intraocular injection, although other modes of administration may be effective, if the sufficient amount of the compound achieves contact with the vitreous cavity. Intraocular injection may be effected by intravitreal injection, aqueous humor injection or injection into the external layers of the eye, such as subconjunctival injection or subtenon injection, or by topical application to the cornea, if a penetrating formulation is used.

Administration

The compositions containing the compounds are administered intraocularly or by other means, such as topically in the form of penetrating eyedrops, whereby contact of the recombinant vectors with the aqueous humor is effected. Intraocular administration may be effected by intravitreal injection, aqueous humor injection, injection into the external layers of the eye, such as

subconjunctival injection or subtenon injection, preferably in free form, but, alternatively, in liposomes or other sustained drug delivery device. Administration is preferably by intravitreal injection, preferably through self sealing 25-30 gauge needles or other suitably calibrated delivery device. Injection into the eye may be through the pars plana via the self-sealing needle.

It is further understood that, for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the recombinant viruses, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed methods

F. Diseases, Disorders and therapeutic products

1. Disease and disorders

Retinitis pigmentosa

Methods for specifically or selectively targeting recombinant adenovirus vectors for delivery of gene products, particularly therapeutic products are provided herein. These methods are particularly suitable for targeting cells that express receptors that are selectively recognized by Ad virus of subgroup D viruses, particularly Ad37. It is shown herein that these viruses selectively recognize receptors on cells, such as conjunctival cells and photoreceptors, that are not recognized by other adenoviruses. Hence, methods for targeting to these cell types by providing vectors that are packaged in viral particles that contain a sufficient portion of a fiber protein from one of these Ad serotypes to bind to these receptors. These methods are useful for targeting to photoreceptors and for treating ocular disorders, including, but are not limited to, inherited and acquired retinal, neovascular degenerative diseases (see table below).

It is estimated that 1 in 3,500 individuals in the United States suffer from one of the pigmented retinopathies. This group of retinal diseases, commonly called retinitis pigmentosa, is characterized by progressive loss of peripheral and night vision. Patients may be affected at almost any age and it is not uncommon to experience symptoms in early childhood in certain inherited forms.

It has been shown that there are a variety of mutations in genes expressed in the photoreceptors, including genes in the rhodopsin gene and pathway that appear to be responsible for these diseases. In addition to mutations in rhodopsin, changes in the retinal pigmented epithelial (RPE) cells, also undergo degenerative changes and can form clumps of pigment that give rise to the characteristic pigmentary changes seen in patients with RP.

Angiogenesis and ocular diseases and disorders

The vast majority of diseases that cause catastrophic loss of vision do so as a result of ocular neovascularization; age related macular degeneration (ARMD) affects 12-15 million American over the age of 65 and causes visual loss in 10-15% of them as a direct effect of choroidal (sub-retinal) neovascularization. The leading cause of visual loss for Americans under the age of 65 is diabetes; 16 million individuals in the United States are diabetic and 40,000 per year suffer from ocular complications of the disease, which often are a result of retinal neovascularization. Laser photocoagulation has been effective in preventing severe visual loss in subgroups of high risk diabetic patients, but the overall 10 year incidence of retinopathy remains essentially unchanged. For patients with choroidal neovascularization due to ARMD or inflammatory eye disease, such as ocular histoplasmosis, photocoagulation, with few exceptions, is ineffective in preventing visual loss. While recently developed, non-destructive photodynamic therapies hold promise for temporarily reducing individual loss in patients with previously untreatable choroidal neovascularization, only 61.4% of patients treated every 3-4 months had improved or stabilized vision compared to 45.9% of the placebo-treated group.

In the normal adult, angiogenesis is tightly regulated and limited to wound healing, pregnancy and uterine cycling. Angiogenesis is turned on by specific angiogenic molecules such as basic and acidic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor (TGF), tumor necrosis factor-α (TNF-α) and platelet derived growth factor (PDGF). Angiogenesis can be suppressed by inhibitory molecules such as interferon-α, thrombospondin-1, angiostatin and endostatin. It is the balance of these naturally occurring stimulators and inhibitors that controls the normally

quiescent capillary vasculature. When this balance is upset, as in certain disease states, capillary endothelial cells are induced to proliferate, migrate and ultimately differentiate.

Angiogenesis plays a central role in a variety of diseases, including, but are not limited to, cancer and ocular neovascularization. Sustained growth and metastasis of a variety of tumors has also been shown to be dependent on the growth of new host blood vessels into the tumor in response to tumor derived angiogenic factors. Proliferation of new blood vessels in response to a variety of stimuli occurs as the dominant finding in the majority of eye diseases that blind, such as, but are not limited to, proliferative diabetic retinopathy (PDR), ARMD, rubeotic glaucoma, interstitial keratitis and retinopathy of prematurity. In these diseases, tissue damage can stimulate release of angiogenic factors resulting in capillary proliferation. VEGF plays a dominant role in iris neovascularization and neovascular retinopathies. While reports clearly show a correlation between intraocular VEGF levels and ischemic retinopathic ocular neovascularization, FGF likely plays a role. Basic and acidic FGF are known to be present in the normal adult retina, even though detectable levels are not consistently correlated with neovascularization. This may be largely due to the fact that FGF binds very tightly to charged components of the extracellular matrix and may not be readily available in a freely diffusible form that would be detected by standard assays of intraocular fluids.

A final common pathway in the angiogenic response involves integrin-mediated information exchange between a proliferating vascular endothelial cell and the extracellular matrix. This class of adhesion receptors, called integrins, are expressed as heterodimers having an α and β subunit on all cells. One such integrin, $\alpha_v\beta_3$, is the most promiscuous member of this family and allows endothelial cells to interact with a wide variety of extracellular matrix components. Peptide and antibody antagonists of this integrin inhibit angiogenesis by selectively inducing apoptosis of the proliferating vascular endothelial cells. Two cytokine-dependent pathways of angiogenesis exist and may be defined by their dependency on distinct vascular cell integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Specifically, basic FGF- and VEGF-induced angiogenesis depend on integrin

$\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively, since antibody antagonists of each integrin selectively block one of these angiogenic pathways in the rabbit corneal and chick chorioallantoic membrane (CAM) models. Peptide antagonists that block all α_v integrins inhibit FGF- and VEGF-stimulated angiogenesis. While normal human ocular blood vessels do not display either integrin, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are selectively displayed on blood vessels in tissues from patients with active neovascular eye disease. While only $\alpha_v\beta_3$ was consistently observed in tissue from patients with ARMD,$\alpha_v\beta_3$ and $\alpha_v\beta_5$ were present in tissues from patients with PDR. Systemically administered peptide antagonists of integrins blocked new blood vessel formation in a mouse model of retinal vasculogenesis.

In addition to adhesion events described above, cell migration through the extracellular matrix also depends on proteolysis. Matrix metalloproteinases are a family of zinc-requiring matrix-degrading enzymes that include the collagenases, gelatinases and stromelysins, all of which have been implicated in invasive cell behavior. Invasive cell processes such as tumor metastasis and angiogenesis have been found to be associated with the expression of integrins and MMP-2, MMP-2 are all found throughout the eye where they may interact to maintain a quiescent vasculature until the balance is upset, resulting in pathological angiogenesis. A non-catalytic C-terminal hemopexin-like domain of MMP-2 (PEX) can block cell surface collagenolytic activity and inhibit angiogenesis in the CAM model by preventing localization of MMP-2 to the surface of invasive cells through interaction with the integrin $\alpha_v\beta_3$.

Hence, anti-angiogenic agents have a role in treating retinal degeneration to prevent the damaging effects of these trophic and growth factors. Angiogenic agents, also have a role in promoting desirable vascularization to retard retinal degeneration by enhancing blood flow to cells.

Members of adenovirus subgroup D, Ad8, 19A, and 37, are infectious agents that cause particularly severe cases of epidemic keratoconjunctivitis (EKC) (Arnberg *et al.* (1998) *Virology 227*:239-244; Curtis *et al.* (1998) *J.Med.Microbiol. 47*:91-94; Ritterband *et al.* (1998) *Rev.Med.Virol. 8*:187-201; and Takeuchi *et al.* (1999) *J.Clin.Microbiol. 37*:3392-3394). There is no effective treatment for this debilitating and contagious disease and EKC

continues to be a problem in ophthalmology clinics worldwide (Curtis *et al.* (1998) *J.Med.Microbiol.* *47*:91-94, Lukashok *et al.* (1998) *Curr.Clin.Top.Infec.Dis.* *18*:286-304). Hence the vectors herein may be used for treating the disease.

Table 3
Candidate targets for ocular disease therapy

CANDIDATE TARGETS FOR OCULAR DISEASE THERAPY	
Disease	**Candidate target(s)**
Retinitis pigmentosa	Rhodopsin gene, and genes that regulate expression thereof *rds*/peripherin
Stargardt's disease	rim protein (ARC protein)
Choroideremia	rab geranylgeranyl transferase CHM, TCD, CHML*
Gyrate Atrophy	ornithine aminotransferase
Macular dystrophy	*rds*/peripherin

* see, "MSR6-yeast homologue of the choroideraemia gene," Nature Genetics 3: 193-4 (1993)

TABLE 4

Other Diseases
Exudative Choroidal Diseases
ICSC, fluorescein angiogram
ICSC with large serious detachment of RPE (retinal pigmented epithelium)
ICSC with bullous retinal detachment
Macular drusen, exudative, confluent
Drusen, sub-RPE choroidal neovascularization
Drusen, notched serous detachment of RPE

Other Diseases
Drusen, notched serous and hemorrhagic detachment of RPE
Drusen, serous and hemorrhagic detachment of RPE and retina
Drusen, organized RPE detachment causing bullous retinal detachment
Drusen, geographic atrophy of RPE
Drusen, exudative and cuticular, vitelliform macular detachment
Drusen, cuticular, large vitelliform macular detachment
North Carolina dystrophy with macular staphyloma
North Carolina dystrophy with macular staphyloma
Angioid streaks, pseudoxanthoma elasticum (PXE), CNVM
Angioid streaks, PXE, large notched retinal detachment
Myopic degeneration, Foerster-Fuchs spot
Presumed ocular histoplasmosis syndrome (POHS)
Submacular bacterial abscess
Toxocara canis, subretinal granuloma
Serpiginous (geographic) choroiditis
Posterior scleritis
Harada's disease
Posterior sympathetic uveitis
Benign reactive lymphoid hyperplasia of uveal tract
Choroidal ruptures and CNVM
Cavernous hemangioma of choroid
Choroidal osteoma
Choroidal nevus, serous macular detachment
Choroidal nevus with CNVM
Diffuse sclerochoroidal melanocytic nevus
Choroidal melanoma with serous detachment of RPE
Metastatic lung carcinoma to choroid
Sub-RPE reticulum cell sarcoma

Other Diseases
RPE tear, idiopathic choroidal neovascularization
Heredodystrophic Disorders Affecting RPE & Retina
Best's vitelliform macular dystrophy
Best's vitelliform macular dystrophy with CNVM
Best's vitelliform macular dystrophy, multiple lesions
Adult-onset vitelliform foveomacular dystrophy
Pattern dystrophy simulating fundus flavimaculatus
Stargardt's disease (fundus flavimaculatus)
Asteroid macular dystrophy
Sjögren-Larssen syndrome
Oguchi's disease, light-adapted state
Oguchi's disease, dark-adapted state
Fundus albipunctatus
Retinitis pigmentosa, cystoid macular edema
Crystalline tapetoretinal dystrophy
Choroideremia
Goldmann-Favre syndrome
Sex-linked juvenile retinoschisis
Perivenous retinitis pigmentosa
Retinal Vascular Disorders
Retinal arteriovenous aneurysm
Central retinal artery occlusion
Cilioretinal artery obstruction
Ischemic retinopathy in systemic lupus erythematosus
Ischemic retinopathy in scleroderma
Hemorrhagic detachment of internal limiting membrane, hypertensive retinopathy
Acquired retinal arterial macroaneurysm

Other Diseases
Cystoid macular edema, aphakic
Cystoid macular edema, nicotinic acid maculopathy
Congenital retinal telangiectasis
Acquired bilateral juxtafoveal telangiectasis
Acquired bilateral juxtafoveal obliterative telangiectasis
Diabetic optic neuropathy
X-ray radiation exudative retinopathy
Sickle cell SC disease, macular hemorrhage
Retinal arterial aneurysms, arteritis, neuroretinitis
Branch retinal vein obstruction (BRVO)
BRVO, exudative maculopathy
BRVO, optic disc new vessels, photocoagulation
Waldenström's macroglobulinemia
Inflammatory Diseases of the Retina and Choroid
Luetic retinal vasculitis
Focal *Candida* retinal abscess
Toxoplasmosis, atrophic chorioretinal scar
Toxoplasmosis retinitis and macular detachment
Toxoplasmosis scar, CNVM, macular detachment
Diffuse unilateral subacute neuroretinitis, small worm
Diffuse unilateral subacute neuroretinitis, large worm
Cytomegalic inclusion disease, papillitis
Acute posterior multifocal placoid pigment epitheliopathy
Acute macular neuroretinitis
Sarcoid retinitis
Sarcoid papillitis
Behcet's disease
Vitiliginous (bird-shot) chorioretinitis

Other Diseases
Multifocal choroiditis and panveitis (pseudo-POHS)
Retinal and Pigment Epithelial Hamartomas
Congenital grouped albinotic RPE spots
Congenital hyperplasia of RPE
Combined RPE and retinal hamartoma, juxtapapillary
Combined RPE and retinal hamartoma, peripheral
Cystic astrocytoma, juxtapapillary
Astrocytoma, macula
Astrocytoma, juxtapapillary
Cavernous hemangioma of retina
Juxtapapillary sessile retinal capillary hemangioma
Juxtapapillary endophytic retinal capillary hemangioma
Other Tumors of the Choroid
Choroidal metastasis
Choroidal osteoma
Choroidal hemangioma
Miscellaneous uveal tumors
Intraocular Lymphoid Tumors
The leukemias and lymphomas
Tumors of the Vitreous
Non-Hodgkins ("reticulum cell") lymphoma
Tumor involvement of the vitreous cavity
Macular Disease
Age-related macular degeneration -- atrophic form
Exudative age-related macular degeneration
Choroidal neovascular membrane in degenerative myopia
Central serous retinopathy
Macular hole

Other Diseases
Macular dystrophies
Retinal Vascular Disease
Etiologic mechanisms in diabetic retinopathy
Background diabetic retinopathy
Proliferative diabetic retinopathy
Retinal arterial obstructive disease
Central retinal vein occlusion
Retinal branch vein occlusion
Pregnancy and retinal disease
Pregnancy-induced hypertension
Hypertension
The rheumatic disease
Parafoveal telangiectasis
Coats disease
Disseminated intravascular systemic coagulopathy and related vasculopathies
Hemoglobinopathies
Retinopathy of prematurity
Acquired retinal macroaneurysms
Eales disease
Radiation retinopathy
The ocular ischemic syndrome
Inflammatory Disease
Ocular toxoplasmosis
Ocular toxocariasis
Ocular cysticercosis
Cytomegalovirus infections of the retina
Retinal and ophthalmologic manifestations of AIDS
Acute retinal necrosis syndrome

Other Diseases
Endogenous fungal infections of the retina and choroid
Pars planitis
Syphilis and tuberculosis
Diffuse unilateral subacute neuroretinitis
Scleritis
Birdshot retinochoroidopathy
Punctate inner choroidopathy
Sarcoidosis
Acute multifocal placoid pigment epitheliopathy
Geographic helicoid peripapillary choroidopathy (GHPC): serpiginous choroiditis
Sympathetic ophthalmia
Vogt-Koyanigi-Harada syndrome (uveomeningitic syndrome)
Ciliochoroidal (uveal) effusion

Reproduced from: Stereoscopic Atlas of Ocular Diseases Diagnosis and Treatment, 2nd Edition, J. Donald O. Gass, Vol. 1 & 2, C.V. Mosley Co. (1987); and Retina Vol. II, Editor, Stephen J. Ryan, Medical Retina, C.V. Moslay Co. (1989).

2. **Therapeutic products**

Therapeutic products include but are not limited to, wild-type genes that are defective in ocular disorders, such as rhodopsin, or fragments thereof sufficient to correct the genetic defect, trophic factors, including growth factors, inhibitors and agonists of trophic factors, anti-apoptosis factors and other products described herein or known to those of skill in the art to be useful for treatment of disorders of the eye or that can be treated by a product expressed by a photoreceptor.

OCULAR GENE THERAPY STRATEGIES		
GENERAL DISEASE	EXAMPLES	STRATEGY

OCULAR GENE THERAPY STRATEGIES		
Hereditary retinal and macular degeneration	• Retinitis pigmentosa • Stargardt's disease • Other macular dystrophies	Growth factors (e.g., GDNF) anti-apoptotic factors (e.g., bcl2 gene) Stargardt Disease Gene (ABCR)[†]
Neovascular	• Diabetes • Choroidal neovascularization	Anti-angiogenesis factors
Anti-tumor	Retinoblastoma	Antiproliferant
Glaucoma	Nerve fiber layer atrophy	Neuroprotective agent

[†] See Allikmets *et al.* (1997) *Science* *277*:1805-1807.

For example, for treatment of retinitis pigmentosa the adenovirus vector can deliver a wild-type rhodopsin gene or a growth factor or trophic factor, such as ciliary neurotrophic factor CNTF; for treatment of Stargardt's disease, the vector can deliver a wild type ABCR (also called STGD1) or a growth factor or anti-angiogenic agent; for diabetic retinopathies, retinal vascularization the vector can deliver growth factors, such as a TGF (TGFβ), to prevent degeneration.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

Preparation of Adenovirus Packaging Cell Lines

Cell lines that are commonly used for growing adenovirus are useful as host cells for the preparation of adenovirus packaging cell lines. Preferred cells include 293 cells, an adenovirus-transformed human embryonic kidney cell line obtained from the ATCC, having Accession Number CRL 1573; HeLa, a human epithelial carcinoma cell line (ATCC Accession Number CCL-2); A549, a human lung carcinoma cell line (ATCC Accession Number CCL 1889); and other epithelial-derived cell lines. As a result of the adenovirus transformation, the

293 cells contain the E1 early region regulatory gene. All cells were maintained in complete DMEM + 10% fetal calf serum unless otherwise noted.

These cell lines allow the production and propagation of adenovirus-based gene delivery vectors that have deletions in preselected gene regions and that are obtained by cellular complementation of adenoviral genes. To provide the desired complementation of such deleted adenoviral genomes in order to generate a viral vector, plasmid vectors that contain preselected functional units have been designed. Such units include but are not limited to E1 early region, E4 and the viral fiber gene. The preparation of plasmids providing such complementation, thereby being "complementary plasmids or constructs," that are stably inserted into host cell chromosomes are described below.

A. Preparation of an E4-Expressing Plasmid for Complementation of E4-Gene-Deleted Adenoviruses

The viral E4 regulatory region contains a single transcription unit that is alternately spliced to produce several different mRNA products. The E4-expressing plasmid prepared as described herein and used to transfect the 293 cell line contains the entire E4 transcription unit. A DNA fragment extending from 175 nucleotides upstream of the E4 transcription start site including the natural E4 promoter to 153 nucleotides downstream of the E4 polyadenylation signal including the natural E4 terminator signal, corresponding to nucleotides 32667-35780 of the adenovirus type 5 (hereinafter referred to as Ad5) genome as described in Chroboczek *et al.* (*Virol.*, 186:280-285 (1992), GenBank Accession Number M73260), was amplified from Ad5 genomic DNA, obtained from the ATCC, via the polymerase chain reaction (PCR). Sequences of the primers used were 5'CGGTACACA<u>GAATTC</u>AGGAGACACAACTCC3' (forward or 5' primer referred to as E4L) (SEQ ID NO: 1) and 5'GCCT<u>GGATCC</u>GGGAAGTTACGTAACGTGGGAAAAC3' (SEQ ID NO: 2) (backward or 3' primer referred to as E4R). To facilitate cloning of the PCR fragment, these oligonucleotides were designed to create new sites for the restriction enzymes EcoRI and BamHI, respectively, as indicated with underlined nucleotides. DNA was amplified via PCR using 30 cycles of 92 C for 1 minute,

50 C for 1 minute, and 72 C for 3 minutes resulting in amplified full-length E4 gene products.

The amplified DNA E4 products were then digested with EcoRI and BamHI for cloning into the compatible sites of pBluescript/SK+ by standard techniques to create the plasmid pBS/E4. A 2603 base pair (bp) cassette including the herpes simplex virus thymidine kinase promoter, the hygromycin resistance gene, and the thymidine kinase polyadenylation signal was excised from the plasmid pMEP4 (Invitrogen, San Diego, CA) by digestion with FspI followed by addition of BamHI linkers (5'CGCGGATCCGCG3') (SEQ ID NO: 3) for subsequent digestion with BamHI to isolate the hygromycin-containing fragment.

The isolated BamHI-modified fragment was then cloned into the BamHI site of pBS/E4 containing the E4 region to create the plasmid pE4/Hygro containing 8710 bp. The pE4/Hygro plasmid has been deposited with the ATCC under accession number 97739. The complete nucleotide sequence of pE4/Hygro is set forth in SEQ ID NO: 4. Position number 1 of the linearized vector corresponds to approximately the middle portion of the pBS/SK+ backbone. The 5' and 3' ends of the E4 gene are located at respective nucleotide positions 3820 and 707 of SEQ ID NO: 4 while the 5' and 3' ends of the hygromycin insert are located at respective nucleotide positions 3830 and 6470. In the clone that was selected for use, the E4 and hygromycin resistance genes were divergently transcribed.

B. Preparation of a Fiber-Expressing Plasmid for Complementation of Fiber-Gene-Deleted Adenoviruses

To prepare a fiber-encoding construct, primers were designed to amplify the fiber coding region from Ad5 genomic DNA with the addition of unique BamHI and NotI sites at the 5' and 3' ends of the fragment, respectively. The Ad5 nucleotide sequence is available with the GenBank Accession Number M18369. The 5' and 3' primers had the respective nucleotide sequences of 5'ATG<u>GGATCC</u>AAGATGAAGCGCGCAAGACCG3' (SEQ ID NO: 5) and 5'CATAAC<u>GCGGCCGC</u>TTCTTTATTCTTGGGC3' (SEQ ID NO: 6), where the inserted BamHI and NotI sites are indicated by underlining. The 5' primer also

contained a nucleotide substitution 3 nucleotides 5′ of the second ATG codon (C to A) that is the initiation site. The nucleotide substitution was included so as to improve the consensus for initiation of fiber protein translation.

The amplified DNA fragment was inserted into the BamHI and NotI sites of pcDNA3 (Invitrogen) to create the plasmid designated pCDNA3/Fiber having 7148 bp. The parent plasmid contained the CMV promoter, the bovine growth hormone (BHG) terminator and the gene for conferring neomycin resistance. The viral sequence included in this construct corresponds to nucleotides 31040-32791 of the Ad5 genome.

The complete nucleotide sequence of pCDNA3/Fiber is listed in SEQ ID NO: 7 where the nucleotide position 1 corresponds to approximately the middle of the pcDNA3 vector sequence. The 5′ and 3′ ends of the fiber gene are located at respective nucleotide positions 916 with ATG and 2661 with TAA.

To enhance expression of fiber protein by the constitutive CMV promoter provided by the pcDNA vector, a BglII fragment containing the tripartite leader (TPL) of adenovirus type 5 was excised from pRD112a (Sheay *et al.*, *BioTechniques*, 15:856-862 (1993) and inserted into the BamHI site of pCDNA3/Fiber to create the plasmid pCLF having 7469 bp. The adenovirus tripartite leader sequence, present at the 5′ end of all major late adenoviral mRNAs as described by Logan *et al.*, *Proc. Natl. Acad. Sci., USA*, 81:3655-3659 (1984) and Berkner, *BioTechniques, 6*:616-629 (1988), also referred to as a "partial TPL" since it contains a partial exon 1, shows correspondence with the Ad5 leader sequence having three spatially separated exons corresponding to nucleotide positions 6081-6089 (the 3′ end of the first leader segment), 7111-7182 (the entire second leader segment), and 9644-9845 (the third leader segment and sequence downstream of that segment). The corresponding cDNA sequence of the partial tripartite leader sequence present in pCLF is included in SEQ ID NO: 8 bordered by BamHI/BglII 5′ and 3′ sites at respective nucleotide positions 907-912 to 1228-1233. The nucleotide sequence of an isolated partial TPL is also listed separately as SEQ ID No. 22 with the noted 5′ and 3′ restriction sites and with the following nucleotide regions identified: 1-6 nt BglII site; 1-18 nt polylinker; 19-27 nt last 9 nt of the first leader segment (exon 1);

28-99 nt second leader segment (exon 2); 100-187 nt third leader segment (exon 3); 188-301 nt contains the nt sequence immediately following the third leader in the genome with an unknown function; and 322-327 nt BglII site.

The pCLF plasmid has been deposited with the ATCC as described in Example 4. The complete nucleotide sequence of pCLF is listed in SEQ ID NO: 8 where the nucleotide position 1 corresponds to approximately the middle of the pcDNA3 parent vector sequence. The 5' and 3' ends of the Ad5 fiber gene are located at respective nucleotide positions 1237-1239 with ATG and 2980-2982 with TAA.

C. Generation of an Adenovirus Packaging Cell Line Carrying Plasmids Encoding Functional E4 and Fiber Proteins

The 293 cell line was selected for preparing the first adenovirus packaging line as it already contains the E1 gene as prepared by Graham *et al.*, *J. Gen. Virol.*, *36*:59-74 (1977) and as further characterized by Spector, *Virol.*, *130*:533-538 (1983). Before electroporation, 293 cells were grown in RPMI medium + 10% fetal calf serum. Four x 106 cells were electroporated with 20 μg each of pE4/Hygro DNA and pCLF DNA using a BioRad GenePulser and settings of 300 V, 25 μF. DNA for electroporation was prepared using the Qiagen system according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

Following electroporation, cells were split into fresh complete DMEM + 10% fetal calf serum containing 200 μg/ml Hygromycin B (Sigma, St. Louis, MO).

From expanded colonies, genomic DNA was isolated using the "MICROTURBOGEN" system (Invitrogen) according to manufacturer's instructions. The presence of integrated E4 DNA was assessed by PCR using the primer pair E4R and ORF6L (5'TGCTTAAGCGGCCGCGAAGGAGA AGTCC3') (SEQ ID NO: 9), the latter of which is a 5' forward primer near adenovirus 5 open reading frame 6.

One clone, designated 211, was selected exhibiting altered growth properties relative to that seen in parent cell line 293. The 211 clone contained the product,indicating the presence of inserted DNA corresponding to most, if

not all, of the E4 fragment contained in the pE4/Hygro plasmid. The 211 cell line has been deposited with the ATCC as described in Example 4. This line was further evaluated by amplification using the primer pair E4L/E4R described above, and a product corresponding to the full-length E4 insert was detected. Genomic Southern blotting was performed on DNA restricted with EcoRI and BamHI. The E4 fragment was then detected at approximately one copy/genome compared to standards with the EcoRI/BamHI E4 fragment as cloned into pBS/E4 for use as a labeled probe with the Genius system according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). In DNA from the 211 cell line, the labeled internal fragment pE4/Hygro hybridized with the isolated E4 sequences. In addition, the probe hybridized to a larger fragment which may be the result of a second insertion event.

Although the 211 cell line was not selected by neomycin resistance, thus indicating the absence of fiber gene, to confirm the lack of fiber gene, the 211 cell line was analyzed for expression of fiber protein by indirect immunofluorescence with an anti-fiber polyclonal antibody and a FITC-labeled anti-rabbit IgG (KPL) as secondary. No immunoreactivity was detected. Therefore, to generate 211 clones containing recombinant fiber genes, the 211 clone was expanded by growing in RPMI medium and subjected to additional electroporation with the fiber-encoding pCLF plasmid as described above.

Following electroporation, cells were plated in DMEM + 10% fetal calf serum and colonies were selected with 200 μg/ml G418 (Gibco, Gaithersburg, MD). Positive cell lines remained hygromycin resistant. These candidate sublines of 211 were then screened for fiber protein expression by indirect immunofluorescence as described above. The three sublines screened, 211A, 211B and 211R, along with a number of other sublines, all exhibited nuclear staining qualitatively comparable to the positive control of 293 cells infected with AdRSVβgal (1 pfu/cell) and stained 24 hours post-infection.

Lines positive for nuclear staining in this assay were then subjected to Western blot analysis under denaturing conditions using the same antibody. Several lines in which the antibody detected a protein of the predicted molecular weight (62 kd for the Ad5 fiber protein) were selected for further study including

211A, 211B and 211R. The 211A cell line has been deposited with ATCC as described in Example 4.

Immunoprecipitation analysis using soluble nuclear extracts from these three cell lines and a seminative electrophoresis system demonstrated that the fiber protein expressed is in the functional trimeric form characteristic of the native fiber protein. The predicted molecular weight of a trimerized fiber is 186 kd. Under denaturing conditions, the trimeric form was destroyed resulting in detectable fiber monomers. Those clones containing endogenous E1, newly expressed recombinant E4 and fiber proteins were selected for use in complementing adenovirus gene delivery vectors having the corresponding adenoviral genes deleted as described in Example 2.

D. Preparation of an E1-Expressing Plasmid for Complementation of E1-Gene-Deleted Adenoviruses

In order to prepare adenoviral packaging cell lines other than those based on the E1-gene containing 293 cell line as described in Example 1C above, plasmid vectors containing E1 alone or in various combinations with E4 and fiber genes are constructed as described below.

The region of the adenovirus genome containing the E1a and E1b gene is amplified from viral genomic DNA by PCR as previously described. The primers used are E1L, the 5' or forward primer, and E1R, the 3' or backward primer, having the respective nucleotide sequences 5'CCG A<u>GCTAGC</u> GACTGAAAATGAG3' (SEQ ID NO: 10) and 5'CCT<u>CTCGAG</u> AGACAGC AAGACAC3' (SEQ ID NO: 11). The E1L and E1R primers include the respective restriction sites NheI and XhoI as indicated by the underlines. The sites are used to clone the amplified E1 gene fragment into the NheI/XhoI sites in pMAM commercially available from Clontech (Palo Alto, CA) to form the plasmid pDEX/E1 having 11152 bp.

The complete nucleotide sequence of pDEX/E1 is listed in SEQ ID NO: 12 where the nucleotide position 1 corresponds to approximately 1454 nucleotides from the 3' end of the pMAM backbone vector sequence. The pDEX/E1 plasmid includes nucleotides 552 to 4090 of the adenovirus genome positioned downstream (beginning at nucleotide position 1460 and ending at 4998 in the

pDEX/E1 plasmid) of the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter of pMAM. The pMAM vector contains the E. coli *gpt* gene that allows stable transfectants to be isolated using hypoxanthine/aminopterin/thymidine (HAT) selection. The pMAM backbone occupies nucleotide positions 1-1454 and 5005-11152 of SEQ ID NO: 12.

E. Generation of an Adenovirus Packaging Cell Line Carrying Plasmids Encoding Functional E1, and Fiber Proteins

To create separate adenovirus packaging cell lines equivalent to that of the 211 sublines, 211A, 211B and 211R, as described in Example 1C, alternative cell lines lacking adenoviral genomes are selected for transfection with the plasmid constructs as described below. Acceptable host cells include A549, Hela, Vero and the like cell lines as described in Example 1. The selected cell line is transfected with the separate plasmids, pDEX/E1and pCLF, respectively for expressing E1, and fiber complementary proteins. Following transfection procedures as previously described, clones containing stable insertions of the two plasmids are isolated by selection with neomycin and HAT. Integration of full-length copy of the E1 gene is assessed by PCR amplification from genomic DNA using the primer set E1L/E1R , as described above. Functional insertion of the fiber gene is assayed by staining with the anti-fiber antibody as previously described.

The resultant stably integrated cell line is then used as a packaging cell system to complement adenoviral gene delivery vectors having the corresponding adenoviral gene deletions as described in Example 2.

F. Preparation of a Plasmid Containing Two or More Adenoviral Genes for Complementing Gene-Deleted Adenoviruses

The methods described in the preceding Examples rely on the use of two plasmids, pE4/Hygro and pCLF, or, pCLF and pDEX/E1 for generating adenoviral cell packaging systems. In alternative embodiments, complementing plasmids containing two or more adenoviral genes for expressing of encoded proteins in various combinations are also prepared as described below. The resultant plasmids are then used in various cell systems with delivery plasmids having the corresponding adenoviral gene deletions. The selection of packaging cell, content of the delivery plasmids and content of the complementing plasmids for

use in generating recombinant adenovirus viral vectors thus depends on whether other adenoviral genes are deleted along with the adenoviral fiber gene, and, if so, which ones.

1. Preparation of a Complementing Plasmid Containing Fiber and E1 Adenoviral Genes

A DNA fragment containing sequences for the CMV promoter, adenovirus tripartite leader, fiber gene and bovine growth hormone terminator is amplified from pCLF prepared in Example 1B using the forward primer 5'GACGGATCGGGAGATCTCC3' (SEQ ID NO: 13), that anneals to the nucleotides 1-19 of the pCDNA3 vector backbone in pCLF, and the backward primer 5'CCGCCTCAGAAGCCATAGAGCC3' (SEQ ID NO: 14) that anneals to nucleotides 1278-1257 of the pCDNA3 vector backbone. The fragment is amplified as previously described and then cloned into the pDEX/E1 plasmid, prepared in Example 1D. For cloning in the DNA fragment, the pDEX/E1 vector is first digested with NdeI, that cuts at a unique site in the pMAM vector backbone in pDEX/E1, then the ends are repaired by treatment with bacteriophage T4 polymerase and dNTPs.

The resulting plasmid containing E1 and fiber genes, designated pE1/Fiber, provides dexamethasone-inducible E1 function as described for DEX/E1 and expression of Ad5 fiber protein as described above.

The complete nucleotide sequence of pE1/Fiber is listed in SEQ ID NO: 15 where the nucleotide position 1 corresponds to approximately 1459 nucleotides from the 3' end of the parent vector pMAM sequence. The 5' and 3' ends of the Ad5 E1 gene are located at respective nucleotide positions 1460 and 4998 followed by pMAM backbone and then separated from the Ad5 fiber from pCLF by the filled-in blunt ended NdeI site. The 5' and 3' ends of the pCLF fiber gene fragment are located at respective nucleotide positions 10922-14223 containing elements as previously described for pCLF.

The resultant pE1/Fiber plasmid is then used to complement one or more delivery plasmids expressing E1 and fiber.

The pE1/Fiber construct is then used to transfect a selected host cell as described in Example 1E to generate stable chromosomal insertions preformed as

previously described followed by selection on HAT medium. The stable cells are then used as packaging cells as described in Example 2.

2. Preparation of a Complementing Plasmid Containing E4 and Fiber Adenoviral Genes

Plasmid pCLF prepared as described in Example 1B is partially digested with BglII to cut only at the site in the pCDNA3 backbone. The pE4/Hygro plasmid prepared in Example 1A is digested with BamHI to produce a fragment containing E4. The E4 fragment is then inserted into the BamHI site of pCLF to form plasmid pE4/Fiber. The resultant plasmid provides expression of the fiber gene as described for pCLF and E4 function as described for pE4/Hygro.

A schematic plasmid map of pE4/Fiber, having 10610 bp. The complete nucleotide sequence of pE4/Fiber is listed in SEQ ID NO: 16 where the nucleotide position 1 corresponds to approximately 14 bp from the 3' end of the parent vector pCDNA3 backbone sequence. The 5' and 3' ends of the Ad5 E4 gene are located at respective nucleotide positions 21 and 3149 followed by fused BglII/BamHI sites and pCDNA3 backbone including the CMV promoter again followed by BglII/BamHI sites. The adenovirus leader sequence begins at nucleotide position 4051 and extends to 4366 followed by fused BamHI/BglII sites and the 5' and 3' ends of the fiber gene located at respective nucleotide positions 4372 and 6124.

Stable chromosonal insertions of pE4/Fiber in host cells are obtained as described above.

EXAMPLE 2

Preparation of Adenoviral Gene Delivery Vectors Using Adenoviral Packaging Cell Lines

Adenoviral delivery vectors are prepared to separately lack the combinations of E1/fiber and E4/fiber. Such vectors are more replication-defective than those previously in use due to the absence of multiple viral genes. A preferred adenoviral delivery vector is replication competent but only via a non-fiber means is one that only lacks the fiber gene but contains the remaining functional adenoviral regulatory and structural genes. Furthermore, these adenovirus delivery vectors have a higher capacity for insertion of foreign DNA.

A. Preparation of Adenoviral Gene Delivery Vectors Having Specific Gene Deletions and Methods of Use

To construct the E1/ fiber deleted viral vector containing the LacZ reporter gene construct, two new plasmids were constructed. The plasmid pΔ E1Bβgal was constructed as follows. A DNA fragment containing the SV40 regulatory sequences and *E. coli* β-galactosidase gene was isolated from pSVβgal (Promega) by digesting with VspI, filling the overhanging ends by treatment with Klenow fragment of DNA polymerase I in the presence of dNTP's and digesting with Bam H1. The resulting fragment was cloned into the EcoRV and BamHI sites in the polylinker of pΔ E1sp1B (Microbix Biosystems, Hamilton, Ontario) to form pΔ E1B βgal that therefore contained the left end of the adenovirus genome with the Ela region replaced by the LacZ cassette (nucleotides 6690 to 4151) of pSVβ gal. Plasmid DNA may be prepared by the alkaline lysis method as described by Birnboim and Doly, *Nuc. Acids Res.,* 7:1513-1523 (1978) or by the Quiagen method according to the manufacturer's instruction, from transformed cells used to expand the plasmid DNA was then purified by CsCl-ethidium bromide density gradient centrifugation. Alternatively, plasmid DNAs may be purified from *E. coli* by standard methods known in the art (e.g. *see* Sambrook *et al.*)

The second plasmid (pDV44), prepared as described herein, is derived from pBHG10, a vector prepared as described by Bett *et al., Proc. Natl. Acad. Sci., USA*, 91:8802-8806 (1994) (see, also International PCT application No. WO 95/00655) using methods well known to one of skill in the art. This vector is also commercially available from Microbix and and contains an Ad5 genome with the packaging signals at the left end deleted and the E3 region (nucleotides 28133:30818) replaced by a linker with a unique site for the restriction enzyme PacI. An 11.9 kb BamHI fragment, which contains the right end of the adenovirus genome, is isolated from pBHG10 and cloned into the BamHI site of pBS/SK(+) to create plasmid p11.3 having approximately 14,658 bp. The p11.3 plasmid was then digested with PacI and SalI to remove the fiber, E4, and inverted terminal repeat (ITR) sequences.

This fragment was replaced with a 3,4 kb fragment containing the ITR segments and the E4 gene which was generated by PCR amplification from pBHG10 using the following oligonucleotide sequences:
5' TGTACACCG GATCCGGCGCACACC3' SEQ ID NO: 17; and
5'CACAACGAGCTC AATTAATTAATTGCCACATCCTC3' SEQ ID NO: 18.
These primers incorporated sites for PacI and BamHI. Cloning this fragment into the PacI and blunt ended SalI sites of the p11.3 backbone resulted in a substitution of the fused ITRs, E4 region and fiber gene present in pBHG10, by the ITRs and E4 region alone. The resulting p11.3 plasmid containing the ITR and E4 regions, designated plasmid pDV43a, was then digested with BamHI. This BamHI fragment was then used to replace a BamHI fragment in pBHG10 thereby creating pDV44 in a pBHG10 backbone.

In an alternative approach to preparing pDV44 with an additional subcloning step to facilitate the incorporation of restriction cloning sites, the following cloning procedure was performed. pDV44 as above was constructed by removing the fiber gene and some of the residual E3 sequences from pBHG10 (Microbix Biosystems). As above, to simplify manipulations, the 11.9 kb BamHI fragment including the rightmost part of the Ad5 genome was removed from pBHG10 and inserted into pBS/SK. The resulting plasmid was termed p11.3. The 3.4 kb DNA fragment corresponding to the E4 region and both ITRs of adenovirus type 5 was amplified as described above from pBHG10 using the oligonucleotides listed above and subcloned into the vector pCR2.1 (Invitrogen) to create pDV42. This step is the additional cloning step to facilitate the incorporation of a SalI restriction site. pDV42 was then digested with PacI, which cuts at a unique site (bold type) in one of the PCR primers, and with SalI, which cuts at a unique site in the pCR2.1 polylinker. This fragment was used to replace the corresponding PacI/XhoI fragment of p11.3 (the pBS polylinker adjacent to the Ad DNA fragment contains a unique XhoI site), creating pDV43.

A plasmid designated pDV44 was constructed by replacing the 11.9 kb BamHI fragment of pBHG10 by the analogous BamHI fragment of pDV43. As generated in the first procedure, pDV44 therefore differs from pBHG10 by the

deletion of Ad5 nucleotides 30819:32743 (residual E3 sequences and all but the 3'-most 41 nucleotides of the fiber open reading frame).

Thus, to summarize, the cloning procedures described above result in the production of a fiber-deleted Ad5 genomic plasmid (pDV44) that was constructed by removing the fiber gene and some of the residual E3 sequences from pBHG10. pDV44 contains a wild-type E4 region, but only the last 41 nucleotides of the fiber ORF (this sequence was retained to avoid affecting expression of the adjacent E4 transcription unit). Plasmids pBHG10 and pDV44 contain unpackageable Ad5 genomes, and must be rescued by cotransfection and subsequent homologous recombination with DNA carrying functional packaging signals. In order to generate vectors marked with a reporter gene, either pDV44 or pBHG10 was cotransfected with pΔE1Bßgal, which contains the left end of the Ad5 genome with an SV40-driven ß-galactosidase reporter gene inserted in place of the E1 region.

In general, and as described below, the method for virus production by recombination of plasmids followed by complementation in cell culture involves the isolation of recombinant viruses by cotransfection of any one of the adenovirus packaging cell systems prepared in Example 1, namely 211A, 211B, 211R, A549, Vero cells, and the like, with plasmids carrying sequences corresponding to viral gene delivery vectors.

A selected cell line is plated in dishes and cotransfected with pDV44 and pΔE1Bβ gal using the calcium phosphate method as described by Bett *et al.*, *Proc. Natl. Acad. Sci., USA, 91*:8802-8806 (1994). Recombination between the overlapping adenovirus sequences in the two plasmids leads to the creation of a full-length viral chromosome where pDV44 and pΔE1Bβ gal recombine to form a recombinant adenovirus vector having multiple deletions. The deletion of E1 and of the fiber gene from the viral chromosome is compensated for by the sequences integrated into the packaging cell genome, and infectious virus particles are produced. The plaques thus generated are isolated and stocks of the recombinant virus are produced by standard methods.

Because of the fiber deletion, a pDV44-derived virus is replication-defective, cells in which it is grown must complement this defect.

The 211B cell line (a derivative of 293 cells which expresses the wild-type (wt) AD5 fiber and is equivalent to 211A on deposit with ATCC as described in Example 4) was used for rescue and propagation of the virus described here. pDV44 and pΔE1ßgal were cotransfected into 211B cells, and the monolayers were observed for evidence of cytopathic effect (CPE). Briefly, for virus construction, cells were transfected with the indicated plasmids using the Gibco Calcium Phosphate Transfection system according to the manufacturer's instructions and observed daily for evidence of CPE.

One of a total of 58 transfected dishes showed evidence of spreading cell death at day 15. A crude freeze-thaw lysate was prepared from these cells and the resulting virus (termed Ad5.ßgal.ΔF) was plaque purified twice and then expanded. To prepare purified viral preparations, cells were infected with the indicated Ad and observed for completion of CPE. Briefly, at day zero, 211B cells were plated in DMEM plus 10% fetal calf serum at approximately 1 X 10^7 cells/150 cm^2 flask or equivalent density. At day one, the medium was replaced with one half the original volume of fresh DMEM containing the indicated Ad, in this case Ad5.ßgal.ΔF, at approximately 100 particles/cell. At day two, an equal volume of medium was added to each flask and the cells were observed for CPE. Two to five days after infection, cells were collected and virus isolated by lysis via four rapid freeze-thaw cycles. Virus was then purified by centrifugation on preformed 15-40% CsCl gradients (111,000 x g for three hours at 4°C). The bands were harvested, dialyzed into storage buffer (10 mM Tris-pH 8.1, 0.9% NaCl, and 10% glycerol), aliquoted and stored at - 70°C. Purified Ad5.ßgal.ΔF virus particles containing human adenovirus Ad5.ßgal.ΔFgenome (described further below) have been deposited with the ATCC on January 15, 1999 as further described in Example 4.

For viral titering, as necessary in the below Examples, Ad preparations were titered by plaque assay on 211B cells. Cells were plated on polylysine-coated 6 well plates at 1.5 x 10^6 cells/well. Duplicate dilutions of virus stock were added to the plates in 1 ml/well of complete DMEM. After a five hour incubation at 37°C, virus was removed and the wells overlaid with 2

ml of 0.6% low-melting agarose in Medium 199 (Gibco). An additional 1 ml of overlay was added at five day intervals.

As a control, the first-generation virus Ad5.ß gal.wt, which is identical to Ad5.ßgal.ΔF except for the fiber deletion, was constructed by cotransfection of pBHG10 and pΔE1BßgaI. In contrast to the low efficiency of recovery of the fiberless genome (1/58 dishes), all of 9 dishes cotransfected with pΔE1Bβgal and pBHG10 produced virus.

In another embodiment, a delivery plasmid is prepared that does not require the above-described recombination events to prepare a viral vector having a fiber gene deletion. In one embodiment, a single delivery plasmid containing all the adenoviral genome necessary for packaging but lacking the fiber gene is prepared from plasmid pFG140 containing full-length Ad5 that is commercially available from Microbix. The resultant delivery plasmid referred to as pFG140-f is then used with pCLF stably integrated cells as described above to prepare a viral vector lacking fiber. For genetic therapy, the fiber gene can be replaced with a therapeutic gene of interest for preparing a therapeutic delivery adenoviral vector. Methods for producing a fiberless vector with a complete TPL are described in Example 3.

Vectors for the delivery of any desired gene and preferably a therapeutic gene are prepared by cloning the gene of interest into the multiple cloning sites in the polylinker of commercially available pΔE1sp1B (Microbix Biosystems), in an analogous manner as performed for preparing pE1Bβ gal as described above. The same cotransfection and recombination procedure is then followed as described herein to obtain viral gene delivery vectors as further discussed in later Examples.

1. **Characterization of the Ad5.βgal.ΔF Genome**

To confirm that the vector genomes had the proper structures and that the fiber gene was absent from the Ad5.ßgal.ΔF chromosome, the DNA isolated from viral particles was analyzed. Briefly, purified viral DNA was obtained by adding 10 μl of 10 mg/ml proteinase K, 40 μl of 0.5 M EDTA and 50 μl of 10% SDS to 800 μl of adenovirus-containing culture supernatant. The suspension

was then incubated at 55°C for 60 minutes. The solution was then extracted once with
400 μl of a 24:1 mixture of chloroform:isoamyl alchohol. The aqueous phase was then removed and precipitated with sodium acetate/ethanol. The pellet was washed once with 70% ethanol and lightly dried. The pellet was then suspended in 40 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Genomic DNA from Ad5.ßgal.wt and Ad5.ßgal.ΔF produced the expected restriction patterns following digestion with either EcoRI or with NdeI. Southern blotting, performed with standard methods, with labeled fiber DNA as a probe demonstrated the presence of fiber sequence in Ad5.ßgal.wt but not in Ad5.ßgal.ΔF DNA. As a positive control, the blot was stripped and reprobed with labeled E4 sequence. Fiber and E4 sequences were detected by using labeled inserts from pCLF and pE4/Hygro, respectively. E4 signal was readily detectable in both genomes at equal intensities. The complete nucleotide sequence of Ad5.ßgal.ΔF is presented in SEQ ID NO: 23 and is contained in the virus particle on deposit with ATCC.

2. Characterization of the Fiberless Adenovirus Ad5.βgal.ΔF

To verify that Ad5.ßgal.ΔF was fiber-defective, 293 cells (which are permissive for growth of E1-deleted Ad vectors but do not express fiber) were infected with Ad5.ßgal.ΔF or with Ad5.ßgal.wt. Twenty-four hours post infection, the cells were stained with polyclonal antibodies directed either against fiber or against the penton base protein. Cells infected with either virus were stained by the anti-penton base antibody, while only cells infected with the Ad5.ßgal.wt control virus reacted with the anti-fiber antibody. This confirms that the fiber-deleted Ad mutant does not direct the synthesis of fiber protein.

3. Growth of the Fiber-Deleted Ad5.βgal.ΔF Vector in Complementing Cells

Ad5.ßgal.ΔF was found to readily be propagated in 211B cells. As assayed by protein concentration, CsCl-purified stocks of either Ad5.ßgal.ΔF or Ad5.ßgal.wt contained similar numbers of viral particles. The particles appeared to band normally on CsCl gradients. Infectivity of the Ad5.ßgal.ΔF particles was lower than the Ad5.ßgal.wt control, as indicated by an increased particle/PFU ratio. Ad5.ßgal.ΔF was also found to plaque more slowly than the control

virus. When plated on 211B cells, Ad5.ßgal.wt plaques appeared within 5-7 days, while plaques of Ad5.ßgal.ΔF continued to appear until as much as 15-18 days post infection. Despite their slower formation, the morphology of Ad5.ßgal.ΔF plaques was essentially normal.

4. Production of Fiberless Ad5.ßgal.ΔF Particles

As Ad5.ßgal.ΔF represents a true fiber null mutation and its stocks are free of helper virus, the fiber mutant phenotype was readily investigated. A single round of growth in cells (such as 293) which do not produce fiber generating a homogeneous preparation of fiberless Ad allowed for the determination of whether such particles would be stable and/or infectious. Either Ad5.ßgal.wt or Ad5.ßgal.ΔF was grown in 293 or 211B cells, and the resulting particles purified on CsCl gradients as previously described. Ad5.ßgal.ΔF particles were readily produced in 293 cells at approximately the same level as the control virus and behaved similarly on the gradients, indicating that there was not a gross defect in morphogenesis of fiberless capsids.

Particles of either virus contained similar amounts of penton base regardless of the cell type in which they were grown. This demonstrated that fiber is not required for assembly of the penton base complex into virions. The Ad5.ßgal.ΔF particles produced in 293 cells did not contain fiber protein. 211B-grown Ad5.ßgal.ΔF also contained less fiber than the Ad5.ßgal.wt control virus. The infectivities of the different viral preparations on epithelial cells correlated with the amount of fiber protein present. The fiberless Ad particles were several thousand-fold less infectious than the first-generation vector control on a per-particle basis, while infectivity of 211B-grown Ad5.ßgal.ΔF was only 50-100 fold less than that of Ad5.ßgal.wt. These studies confirmed fiber's crucial role in infection of epithelial cells via CAR binding.

5. Composition and Structure of the Fiberless Ad5.ßgal.ΔF Particles

The proteins contained in particles of 293-grown Ad5.ßgal.ΔF were compared to those in Ad5.ßgal.wt, to determine whether proteolysis or particle assembly was defective in this fiber null mutant. The overall pattern of proteins in the fiberless particles was observed to be quite similar to that of a

first-generation vector, with the exception of reduced intensity of the composite band resulting from proteins IIIa and IV (fiber). The fiberless particles also had a reduced level of protein VII. Although substantial amounts of uncleaved precursors to proteins VI, VII, and VIII were not seen, it is possible that the low-molecular weight bands migrating ahead of protein VII represent either aberrantly cleaved viral proteins or their breakdown products.

Cryo-electron microscopy was used to more closely examine the structure of the 293 grown Ad5.ßgal.ΔF and of Ad5ßgal.wt. The fiber, having an extended stalk with a knob at the end, was faintly visible in favorable orientations of wild-type Ad5 particles, but not in images of the fiberless particles. Filamentous material likely corresponding to free viral DNA was seen in micrographs of fiberless particles. This material was also present in micrographs of the first-generation control virus, albeit at much lower levels.

Three-dimensional image reconstructions of fiberless and wild-type particles at ~20 Å resolution showed similar sizes and overall features, with the exception that fiberless particles lacked density corresponding to the fiber protein. The densities corresponding to other capsid proteins, including penton base and proteins IIIa, VI, and IX, were comparable in the two structures. This confirms that absence of fiber does not prevent assembly of these components into virions. The fiber was truncated in the wild-type structure as only the lower portion of its flexible shaft follows icosahedral symmetry. The RGD protrusions on the fiberless penton base were angled slightly inward relative to those of the wild-type structure. Another difference between the two penton base proteins was that there is a ~30 Å diameter depression in the fiberless penton base around the five-fold axis where the fiber would normally sit. The Ad5 reconstructions confirm that capsid assembly, including addition of penton base to the vertices, is able to proceed in the complete absence of fiber.

6. Integrin-Dependent Infectivity of Fiberless Ad5.ßgal.ΔF Particles

While attachment via the viral fiber protein is a critical step in the infection of epithelial cells, an alternative pathway for infection of certain hematopoietic cells has been described. In this case, penton base mediates

binding to the cells (via ß2 integrins) and internalization (through interaction with αv integrins). Particles lacking fiber might therefore be expected to be competent for infection of these cells, even though on a per-particle basis they are several thousand-fold less infectious than normal Ad vectors on epithelial cells.

To investigate this, THP-1 monocytic cells were infected with Ad5.ßgal.wt or with Ad5.ßgal.ΔF grown in the absence of fiber. Infection of THP-1 cells was assayed by infecting 2 x 10^5 cells at the indicated m.o.i. in 0.5 ml of complete RPMI. Forty-eight hours post-infection, the cells were fixed with glutaraldehyde and stained with X-gal, and the percentage of stained cells was determined by light microscopy. The results of the infection assay showed that the fiberless particles were only a few-fold less infectious than first-generation Ad on THP-1 cells. Large differences were seen in plaquing efficiency on epithelial (211B) cells. Infection of THP-1 cells by either Ad5.ßgal.ΔF or Ad5.ßgal.wt was not blocked by an excess of soluble recombinant fiber protein, but could be inhibited by the addition of recombinant penton base). These results indicate that the fiberless Ad particles use a fiber-independent pathway to infect these cells. Furthermore, the lack of fiber protein did not prevent Ad5.ßgalΔF from internalizing into the cells and delivering its genome to the nucleus, demonstrating that fiberless particles are properly assembled and are capable of uncoating.

The foregoing results with the recombinant viruses thus produced indicates that they can be used as gene delivery tools in cultured cells and *in vivo* as described more fully in the Examples. For example, for studies of the effectiveness and relative immunogenicity of multiply-deleted vectors, virus particles are produced by growth in the packaging lines described in Example 1 and are purified by CsCl gradient centrifugation. Following titering, virus particles are administered to mice via systemic or local injection or by aerosol delivery to lung. The LacZ reporter gene allows the number and type of cells which are successfully transduced to be evaluated. The duration of transgene expression is evaluated in order to determine the long-term effectiveness of treatment with multiply-deleted recombinant adenoviruses relative to the

standard technologies which have been used in clinical trials to date. The immune response to the improved vectors described here is determined by assessing parameters such as inflammation, production of cytotoxic T lymphocytes directed against the vector, and the nature and magnitude of the antibody response directed against viral proteins.

Versions of the vectors which contain therapeutic genes such as CFTR for treatment of cystic fibrosis or tumor suppressor genes for cancer treatment are evaluated in the animal system for safety and efficiency of gene transfer and expression. Following this evaluation, they are used as experimental therapeutic agents in human clinical trials.

B. Retargeting of Adenoviral Gene Delivery Vectors by Producing Viral Particles Containing Different or Altered Fiber Proteins

As the specificity of adenovirus binding to target cells is largely determined by the fiber protein, viral particles that incorporate modified fiber proteins or fiber proteins from different adenoviral serotypes (pseudotyped vectors) have different specificities. Thus, the methods of expression of the native Ad5 fiber protein in adenovirus packaging cells as described above is also applicable to production of different fiber proteins.

Chimeric fiber proteins can be produced according to known methods (see, *e.g.*, Stevenson *et al.* (1995) *J. Virol., 69*:2850-2857). Determinants for fiber receptor binding activity are located in the head domain of the fiber and an isolated head domain is capable of trimerization and binding to cellular receptors. The head domains of adenovirus type 3 (Ad3) and Ad5 were exchanged in order to produce chimeric fiber proteins. Similar constructs for encoding chimeric fiber proteins for use in the methods herein are contemplated. Thus, instead of the using the intact Ad5 fiber-encoding construct prepared in above and in U.S. application Serial No. 09/482,682) as a complementing viral vector in adenoviral packaging cells, the constructs described herein are used to transfect cells along with E4 and/or E1-encoding constructs.

Briefly, full-length Ad5 and Ad3 fiber genes were amplified from purified adenovirus genomic DNA as a template. The Ad5 and Ad3 nucleotide sequences are available with the respective GenBank Accession Numbers

M18369 and M12411. Oligonucleotide primers are designed to amplify the entire coding sequence of the full-length fiber genes, starting from the start codon, ATG, and ending with the termination codon TAA. For cloning purposes, the 5' and 3' primers contain the respective restriction sites BamHI and NotI for cloning into pcDNA plasmid as described in Example 1A. PCR is performed as described above.

The resulting products are then used to construct chimeric fiber constructs by PCR gene overlap extension (Horton *et al.* (1990) *BioTechniques, 8*:525-535). The Ad5 fiber tail and shaft regions (5TS; the nucleotide region encoding amino acid residue positions 1 to 403) are connected to the Ad3 fiber head region (3H; the nucleotide region encoding amino acid residue positions 136 to 319) to form the 5TS3H fiber chimera. Conversely, the Ad3 fiber tail and shaft regions (3TS; the nucleotide region encoding amino acid residues positions 1 to 135) are connected to the Ad5 fiber head region (5H; the nucleotide region encoding the amino acid residue positions 404 to 581) to form the 3TS5H fiber chimera. The fusions are made at the conserved TLWT (SEQ ID NO: 19) sequence at the fiber shaft-head junction.

The resultant chimeric fiber PCR products are then digested with BamHI and NotI for separate directional ligation into a similarly digested pcDNA 3.1. The TPL sequence is then subcloned into the BamHI as described in Example 1A for preparing an expression vector for subsequent transfection into 211 cells as described above or into the alternative packaging cell systems as previously described. The resultant chimeric fiber construct-containing adenoviral packaging cell lines are then used to complement adenoviral delivery vectors as previously described. Other fiber chimeric constructs are obtained with the various adenovirus serotypes using a similar approach.

In an alternative embodiment, the use of modified proteins including with modified epitopes (see, *e.g.*, Michael *et al.* (1995) *Gene Therapy, 2*:660-668 and International PCT application Publication No. WO 95/26412, which describe the construction of a cell-type specific therapeutic viral vector having a new binding specificity incorporated into the virus concurrent with the destruction of the endogenous viral binding specificity). In particular, the authors described the

production of an adenoviral vector encoding a gastrin releasing peptide (GRP) at the 3' end of the coding sequence of the Ad5 fiber gene. The resulting fiber-GRP fusion protein was expressed and shown to assemble functional fiber trimers that were correctly transported to the nucleus of HeLa cells following synthesis.

Similar constructs are contemplated for use in the complementing adenoviral packaging cell systems for generating new adenoviral gene delivery vectors that are targetable, replication-deficient and less immunogenic. Heterologous ligands contemplated for use herein to redirect fiber specificity range from as few as 10 amino acids in size to large globular structures, some of which necessitate the addition of a spacer region so as to reduce or preclude steric hindrance of the heterologous ligand with the fiber or prevent trimerization of the fiber protein. The ligands are inserted at the end or within the linker region. Preferred ligands include those that target specific cell receptors or those that are used for coupling to other moieties such as biotin and avidin.

A preferred spacer includes a short 12 amino acid peptide linker composed of a series of serines and alanine flanked by a proline residue at each end using routine procedures known to those of skill in the art. The skilled artisan will be with the preparation of linkers to accomplish sufficient protein presentation and to alter the binding specificity of the fiber protein without compromising the cellular events that follow viral internalization. Moreover, within the context of this disclosure, preparation of modified fibers having ligands positioned internally within the fiber protein and at the carboxy terminus as described below are contemplated for use with the methods described herein.

The preparation of a fiber having a heterologous binding ligand is prepared essentially as described in the above-cited paper. Briefly, for the ligand of choice, site-directed mutagenesis is used to insert the coding sequence for a linker into the 3' end of the Ad5 fiber construct in pCLF as prepared in Example 1.

The 3' or antisense or mutagenic oligonucleotide encodes a preferred linker sequence of ProSerAlaSerAlaSerAlaSerAlaProGlySer (SEQ ID NO: 20)

followed by a unique restriction site and two stop codons, respectively, to allow the insertion of a coding sequence for a selected heterologous ligand and to ensure proper translation termination. Flanking this linker sequence, the mutagenic oligonucloetide contains sequences that overlap with the vector sequence and allow its incorporation into the construct. Following mutagenesis of the pCLF sequence adding the linker and stop codon sequences, a nucleotide sequence encoding a preselected ligand is obtained, linkers corresponding to the unique restriction site in the modified construct are attached and then the sequence is cloned into linearized corresponding restriction site. The resultant fiber-ligand construct is then used to transfect 211 or the alternative cell packaging systems previously described to produce complementing viral vector packaging systems.

In a further embodiment, intact fiber genes from different Ad serotypes are expressed by 211 cells or an alternative packaging system as previously described. A gene encoding the fiber protein of interest is first cloned to create a plasmid analogous to pCLF, and stable cell lines producing the fiber protein are generated as described above for Ad5 fiber. The adenovirus vector described which lacks the fiber gene is then propagated in the cell line producing the fiber protein relevant for the purpose at hand. As the only fiber gene present is the one in the packaging cells, the adenoviruses produced contain only the fiber protein of interest and therefore have the binding specificity conferred by the complementing protein. Such viral particles are used in studies such as those described above to determine their properties in experimental animal systems.

EXAMPLE 3

Tripartite leader sequences (TPLs) that are useful in enhancing the expression of complementing adenoviral proteins, particularly fiber protein, for use in preparing an adenoviral gene delivery vector are provided. The complete Ad5 TPL was constructed by assembling PCR fragments. First, the third TPL exon (exon 3) (nt 9644-9731 of the Ad5 genome) was amplified from Ad5 genomic DNA using the synthetic oligonucleotide primers 5'CTCAACAATTGTGGATCCGTACTCC3'(SEQ ID No. 24) and 5'GTGCTCAGCAGATCTTGCGACTGTG3' (SEQ ID No. 25). The resulting

product was cloned to the BamHI and BglII sites of pΔE1Sp1a (Microbix Biosystems) using sites in the primers (shown in bold) to create plasmid pDV52. A fragment corresponding to the first TPL exon (exon 1), the natural first intron (intron 1), and the second TPL exon (exon 2) (Ad5 nt 6049-7182) was then amplified using primers 5'GGCGCGTTCGGATCCACTCTCTTCC3' (SEQ ID No. 26) and 5'CTACATGCTAGGCAGATCTCGTTCGGAG3' (SEQ ID No. 27),and cloned into the BamHI site of pDV52 (again using sites in the primers) to create pDV55.

This plasmid contains a 1.2 kb BamHI/BglII fragment containing the first TPL exon, the natural first intron, and the fused second and third TPL exons. The nucleotide sequence of the complete TPL containing the noted 5' and 3' restriction sites is shown in SEQ ID No 28 with the following nucleotide regions identified: 1-6 nt BamHI site; 7-47 nt first leader segment (exon 1); 48-1068 nt natural first intron (intron 1); 1069-1140 nt second leader segment (exon 2); 1141-1146 nt fused BamHI and BglII sites; 1147-1234 nt third leader segment (exon 3); and 1235-1240 nt BglII site.

EXAMPLE 4

Deposit of Materials

The following cell lines and plasmids were deposited on September 25, 1996, with the American Type Culture Collection, 10801 University Blvd, Manassas, Virginia, USA (ATCC) under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty):Plasmid pE4/Hygro (accession number 97739), Plasmid pCLF (accession number 97737), 211 Cell Line (accession number CRL-12193) and 211A Cell Line (accession number CRL-12194)

The following virus, Ad5.βgal.ΔF, was deposited on January 15, 1999, with the ATCC as listed above and provided with accession number VR2636.

Additionally, plasmids pDV60, pDV67, pDV69, pDV80 and pDV90 were deposited at the ATCC on January 5, 2000 and provided with accession numbers PTA-1144, PTA-1145, PTA-1146, PTA-1147 and PTA-1148 respectively.

EXAMPLE 5

Preparation and Use of Adenoviral Packaging Cell Lines Containing Plasmids Containing Alternative TPLs

Plasmids containing tripartite leaders (TPLs) have been constructed. The resulting plasmids that contain different selectable markers, such as neomycin and zeocin, were then used to prepare fiber-complementing stable cell lines for use as for preparing adenoviral vectors.

A. pDV60

Plasmid pDV60 was constructed by inserting this TPL cassette of SEQ ID No. 28 into the BamHI site upstream of the Ad5 fiber gene in pcDNA3/Fiber, a neomycin selectable plasmid (see, *e.g.*, U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265 on January 14, 2000); see also Von Seggern *et al.*

(1998) *J. Gen Virol., 79*: 1461-1468). The nucleotide sequence of pDV60 is listed in SEQ ID NO: 29. Plasmid pDV60 has been deposited in the ATCC under accession number PTA-1144.

B. pDV61

To construct pDV61, an Asp718/NotI fragment containing the CMV promoter, partial Ad5 TPL, wildtype Ad5 fiber gene, and bovine growth hormone terminator was transferred from pCLF (ATCC accession number 97737; and described in copending U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265 on January 14, 2000);), to a zeocin selectable cloning vector referred to as pCDNA3.1/Zeo (+) (commerically available from Invitrogen and for which the sequence is known).

C. pDV67

In an analogous process, pDV67 containing complete TPL was constructed by transferring an Asp 718/XbaI fragment from pDV60 into pcDNA3.1/Zeo(+) backbone. The nucleotide sequence of pDV67 is set forth in

SEQ ID No. 30. Plasmid pDV67 is available from the ATCC under accession number PTA-1145.

D. pDV69

To prepare pDV69 containing a modified fiber protein, the chimeric Ad3/Ad5 fiber gene was amplified from pGEM5TS3H (Stevenson *et al.* (1995) *J. Virol., 69:* 2850-2857) using the primers 5'ATGGGAT CAAGATGAAGCGCGCAAGACCG3' (SEQ ID No. 31) and 5'CACTATAGCGGCCGCATTCTCAGTCATCTT3' (SEQ ID No. 32), and cloned to the BamHI and NotI sites of pcDNA3.1/Zeo(+) via new BamHI and NotI sites engineered into the primers to create pDV68. Finally, the complete TPL fragment described above was then added to the unique BamH1 site of pDV68 to create pDV69. The nucleotide sequence of pDV69 is listed in SEQ ID No. 33 and has been deposited in the ATCC under accession number PTA-1146.

E. Preparation of Stable Adenovirus Packaging Cell Lines

E1-2a S8 cells are derivatives of the A549 lung carcinoma line (ATCC # CCL 185) with chromosomal insertions of the plasmids pGRE5-2.E1 (also referred to as GRE5-E1-SV40-Hygro construct and listed in SEQ ID No. 34) and pMNeoE2a-3.1 (also referred to as MMTV-E2a-SV40-Neo construct and listed in SEQ ID No. 35), which provide complementation of the adenoviral E1 and E2a functions, respectively. This line and its derivatives were grown in Richter's modified medium (BioWhitaker) + 10% FCS. E1-2a S8 cells were electroporated as previously described (Von Seggern *et al.* (1998) *J. Gen Virol., 79*: 1461-1468) with pDV61, pDV67, or with pDV69, and stable lines were selected with zeocin (600 μg/ml).

The cell line generated with pDV61 is designated 601. The cell line generated with pDV67 is designated 633 while that generated with pDV69 is designated 644. Candidate clones were evaluated by immunofluorescent staining with a polyclonal antibody raised against the Ad2 fiber. Lines expressing the highest level of fiber protein were further characterized.

For the S8 cell complementing cell lines, to induce E1 expression, 0.3 μM of dexamethasone was added to cell cultures 16-24 hours prior to challenge with virus for optimal growth kinetics. For preparing viral plaques, 5 X 10^5

cells/well in 6 well plates are prepared and pre-induced with the same concentration of dexamethasone the day prior to infection with 0.5 μM included at a final concentration in the agar overlay after infection.

F. **Development of Cell Lines for Complementation of E1⁻/E2a⁻ Vectors**

The Adenovirus 5 genome was digested with ScaI enzyme, separated on an agarose gel, and the 6,095 bp fragment containing the left end of the virus genome was isolated. The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the virus is available from the American Type Culture Collection, Manassas, Virginia, U.S.A., under accession number VR-5. The ScaI 6,095 bp fragment was digested further with ClaI at bp 917 and BglII at bp 3,328. The resulting 2,411 bp ClaI to BglII fragment was purified from an agarose gel and ligated into the superlinker shuttle plasmid pSE280 (Invitrogen, San Diego, CA), which was digested with ClaI and BglII, to form pSE280-E.

Polymerase chain reaction (PCR) was performed to synthesize DNA encoding an XhoI and SalI restriction site contiguous with Adenovirus 5 DNA bp 552 through 924. The primers which were employed were as follows:

5′ end, Ad5 bp 552-585:

5′-GTCACTCGAGGACTCGGTC-GACTGAAAATGAGACATATTATCTGCCACGGACC-3′ (SEQ ID No 36)

3′ end, Ad5 bp 922-891:

5′-CGAGATCGATCACCTCCGGTACAAGGTTTGGCATAG-3′ (SEQ ID No. 37)

This amplified DNA fragment (sometimes hereinafter referred to as Fragment A) then was digested with XhoI and ClaI, which cleaves at the native ClaI site (bp 917), and ligated to the XhoI and ClaI sites of pSE280-E, thus reconstituting the 5′ end of the E1 region beginning 8 bp upstream of the ATG codon.

PCR then was performed to amplify Adenovirus 5 DNA from bp 3,323 through 4,090 contiguous with an EcoRI restriction site. The primers which were employed were as follows:

5′ end, Ad5 bp 3323-3360:

5'-CATGAAGATCTGGAAGGTGCTGAGGTACGATGAGACC-3' (SEQ ID No. 38); and

3' end, Ad5 bp 4090-4060:

5'-GCGACTTAAGCAGTCAGCTG-AGACAGCAAGACACTTGCTTGATCCAAATCC -3' (SEQ ID No. 39).

This amplified DNA fragment (sometimes hereinafter referred to as Fragment B) was digested with BglII, thereby cutting at the Adenovirus 5 BglII site (bp 3,382) and EcoRI, and ligated to the BglII and EcoRI sites of pSE280-AE to reconstruct the complete E1a and E1b region from Adenovirus 5 bp 552 through 4,090. The resulting plasmid is designated pSE280-E1.

A construct containing the intact E1a/b region under the control of the synthetic promoter GRE5 was prepared as follows. The intact E1a/b region was excised from pSE280-E1, which was modified previously to contain a BamHI site 3' to the E1 gene, by digesting with XhoI and BamHI. The XhoI to BamHI fragment containing the E1a/b fragment was cloned into the unique XhoI and BamHI sites of pGRE5-2/EBV (U.S. Biochemicals, Cleveland, Ohio) to form pGRE5-E1).

Bacterial transformants containing the final construct were identified. Plasmid DNA was prepared and purified by banding in CsTFA prior to use for transfection of cells.

Construction of plasmid including Adenovirus 5 E2A sequence.

The Adenovirus 5 genome was digested with BamHI and SpeI, which cut at bp 21,562 and 27,080, respectively. Fragments were separated on an agarose gel and the 5,518 bp BamHI to SpeI fragment was isolated. The 5,518 bp BamHI to SpeI fragment was digested further with SmaI, which cuts at bp 23,912. The resulting 2,350 bp BamHI to SmaI fragment was purified from an agarose gel, and ligated into the superlinker shuttle plasmid pSE280, and digested with BamHI and SmaI to form pSE280-E2 BamHI-SmaI.

PCR then was performed to amplify Adenovirus 5 DNA from the SmaI site at bp 23,912 through 24,730 contiguous with NheI and EcoRI restriction sites. The primers which were employed were as follows:

5' end, Ad5 bp 24,732-24,708:

5'-CACGAATTCGTCAGCGCTTCTCGTCGCGTCCAAGACCC-3' (SEQ ID No. 40);
3' end, Ad5 bp 23,912-23,934:
5'-CACCCCGGGGAGGCGGCGGCGACGGGGACGGG-3' (SEQ ID No. 41)

This amplified DNA fragment was digested with SmaI and EcoRI, and ligated to the SmaI and EcoRI sites of pSE280-E2 Bam-Sma to reconstruct the complete E2a region from Ad5 bp 24,730 through 21,562. The resulting construct is pSE280-E2a.

In order to convert the BamHI site at the 3' end of E2a to a SalI site, the E2a region was excised from pSE280-E2a by cutting with BamHI and NheI, and recloned into the unique BamHI and NheI sites of pSE280. Subsequently, the E2a region was excised from this construction with NheI and SalI in order to clone into the NheI and SalI sites of the pMAMneo (Clonetech, Palo Alto, CA) multiple cloning site in a 5' to 3' orientation, respectively. The resulting construct is pMAMneo E2a.

Bacterial transformants containing the final pMAMneo-E2a were identified. Plasmid DNA was prepared and purified by banding in CsTFA. Circular plasmid DNA was linearized at the XmnI site within the ampicillin resistance gene of pMAMneo-E2a, and further purified by the phenol/chloroform extraction and ethanol precipitation prior to use for transfection of cells.

Transfection and selection of cells.

In general, this process involved the sequential introduction, by calcium phosphate precipitation, or other means of DNA delivery, of two plasmid constructions each with a different viral gene, into a single tissue culture cell. The cells were transfected with a first construct and selected for expression of the associated drug resistance gene to establish stable integrants. Individual cell clones were established and assayed for function of the introduced viral gene. Appropriate candidate clones then were transfected with a second construct including a second viral gene and a second selectable marker. Transfected cells then were selected to establish stable integrants of the second construct, and cell clones were established. Cell clones were assayed for functional expression of both viral genes.

A549 (ATCC Accession No. CCL-185) were used for transfection. Appropriate selection conditions were established for G418 and hygromycin B by standard kill curve determination.

Transfection of A549 cells with plasmids including E1 and E2a regions. pMAMNeo-E2a was linearized with XmnI with the Amp^R gene, introduced into cells by transfection, and cells were selected for stable integration of this plasmid by G418 selection until drug resistant colonies arose. The clones were isolated and screened for E2a expression by staining for E2a protein with a polyclonal antiserum, and visualizing by immunofluorescence. E2a function was screened by complementation of the temperature-sensitive mutant Ad5ts125 virus which contains a temperature-sensitive mutation in the E2a gene. (Van Der Vliet, et al., J. Virology, Vol. 15, pgs. 348-354 (1975)). Positive clones expressing the E2a gene were identified and used for transfection with the 7 kb EcoRV to XmnI fragment from pGRE5-E1, which contains the GRE5 promoted E1a/b region plus the $hygromycin^R$ gene. Cells were selected for hygromycin resistance and assayed for E1a/b expression by staining with a monoclonal antibody for the E1 protein (Oncogene Sciences, Uniondale, N.Y.). E1 function was assayed by ability to complement an E1-deleted vector. At this point, expression and function of E2a was verified as described above, thus establishing the expression of E1a/b and E2a in the positive cell clones.

A transfected A549 (A549 (ATCC Accession No. CCL-185);) cell lines showed good E1a/b and E2a expression and was selected for further characterization. It was designated the S8 cell line.

G. Preparation of Adenoviral Vectors Containing Ad5.βgal.ΔF Genome in S8 Improved Fiber-Complementing Cell Lines

To prepare adenoviral vectors containing Ad5.βgal.ΔF (Ad5.βgal.ΔF has been was deposited the ATCC under accession number VR2636) in S8 cells containing alternative forms of TPL for enhancing the expression of fiber proteins, the protocol as described in Example 2 for preparing Ad5.βgal.ΔF in 211B cells was followed with the exception of pretreatment with 0.3 μM dexamethasone for 24 hours as described above. Thus, viral particles with the wildtype Ad5 fiber protein on their surface and containing the fiberless

Ad5.βgal.ΔF genome were produced in 633 cells. Particles produced in 644 cells also contained the fiberless Ad5.βgal.ΔF genome, but had the chimeric 5T3H fiber protein, with the Ad3 fiber knob, on their surface.

Thus, these viral preparations, prepared as described herein are useful for targeting delivery of the Ad5.βgal.ΔF, Ad5.GFP.ΔF, or other similarly constructed fiberless genome with either wild-type or modified fibers. Preferably for purposes herein the fibers are from an Ad serotype D virus, more preferably from Ad37.

EXAMPLE 6

Pseudotyping and Infectivity of Recombinant Adenoviral Vectors Produced with Improved Fiber-Complementing Cell Lines

A. Pseudotyping of Ad5.βgal.ΔF

To verify that adenoviral vectors were produced had altered tropisms, viral particles were purified from either 633 or 644 cells and were then Western blotted and probed with a polyclonal rabbit antibody against the Ad2 fiber (which detects the Ad5 and chimeric 5T3H fiber proteins).

B. Infectivity of Cells with 633 or 644 Generated Virus Particles The cell lines, 633 or 644, prepared as described above, were infected with the indicated number of particles/cell of Ad5.βgal.ΔF and virus particles produced. Virus was then used to infect selected cell lines, including 211B, MRC-5 human fibroblasts, A-10 rat aortic endothelial cells, and THP-1 human monocytic cells. Unbound virus was removed by washing the cells and the cells were further incubated at 37°C for 48 hours. Cells were then fixed with glutaraldehyde and stained with X-gal. The percentage of stained cells was then determined by light microscopy where all experiments were done in triplicate.

The results indicated that adenoviral vectors could be retargeted by pseudotyping using packaging cell lines expressing different fiber proteins. Particles containing either fiber were equally infectious on 211B cells, while MRC-5 fibroblasts and THP-1 cells were more readily infected by virus containing the chimeric fiber. The A-10 rat endothelial cells were more readily infected by particles containing the wildtype Ad5 fiber protein.

EXAMPLE 7

Transient Transcomplementation

The ability of adenovirus type 5 (Ad5) to deliver therapeutic genes to cells is mediated by the interaction of the adenoviral fiber protein with the coxsackievirus-adenoviral receptor (CAR). Because a wide-range of cells express CAR, it was thought that it would be difficult to use adenoviruses to deliver genes to specific cell types. A system for testing modified fiber genes to identify tropisms of interest is described in copending U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265 on January 14, 2000). An *in vitro* system has been developed that involves infection of tissue culture cells with a fiber-deleted Ad and transient co-transfection with a plasmid directing fiber expression. This system allows one to produce and evaluate modified fibers expressed on a viral particle. This system can be used to produce therapeutic quantities of adenoviral vectors with modified fiber proteins, with such fibers having a new tropism added by insertion of a desired ligand into the fiber gene. These fibers may also have the natural tropism (*i.e.* binding to CAR) ablated.

Plasmids used were pDV60 and pDV55 were prepared as described herein and in U.S. application Serial No. 09/482,682 (also filed as International PCT application No. PCT/US00/00265 on January 14, 2000). pDV60 is an pcDNA3.1-based expression plasmid that contains the CMV promoter, Ad5 tripartite leader, an intron, and the Ad5 fiber gene sequence. pDV55 contains no fiber gene and serves as the negative control. Ad5.βgal.ΔF and 211B are described above. 293T cells are identical to 293 cells except they express an integrated SV40 large T antigen gene. HDF cells are human diploid fibroblasts. 293T cells express CAR and α_v integrins; HDF cells express α_v integrins but no CAR. Transfections with fiber expression plasmids were performed with Lipofectamine (GIBCO-BRL) using 20mg DNA and 50ml Lipofectamine per 15cm dish. Cells were maintained in DMEM supplemented with 10% fetal bovine serum.

The fiber deletion mutation of Ad5.βgal.ΔF is complemented in *trans* by passaging virions through 211B, a cell line that stably expresses functional Ad5

fiber. The present system was designed to complement Ad5.βgal.ΔF by modified fibers expressed from transfected episomal plasmids in 293T cells. The result is a simplified and rapid method to incorporate modified fibers on a viral particle containing the Ad5.βgal.ΔF genome that does not require propagation of the virus.

The feasibility of transcomplementation of Ad5.βgal.ΔF with episomal fiber-expressing plasmids was demonstrated in the following experiment. 293T cells were transfected with one of two plasmids: pDV55, which expresses no fiber or pDV60, which expresses wildtype Ad5 fiber. Fiber expression persists for at least six days. Twenty-four hours after transfection, these cells were infected at 2000 particles/cell with Ad5.βgal.ΔF passaged through 211B cells. Seventy-two hours later, a crude viral lysate (CVL) was generated by exposing the cells to five freeze-thaw cycles. Viral particles were purified by cesium chloride gradient centrifugation. The resulting virions incorporated the fiber expressed from the episomal plasmid, as confirmed by Western blots performed with an antibody specific to the Ad5 fiber.

Episomal plasmid transcomplementation system is suitable for quickly expressing and evaluating the properties of modified fibers in the context of a viral particle. Episomal plasmid transcomplementation will also be of great utility for quickly evaluating a bank of modified fibers for other binding properties, including new tropisms and the ablation of the native tropism. In addition to the rapid generation and testing of large numbers of modified fibers, there are other advantages to the Ad5.βgal.ΔF transcomplementation system in terms of production and safety. Episomal plasmid transcomplementation has the inherent advantage over transcomplementation in that it is not necessary to make a stable cell line for every modified fiber for complementation with Ad5.βgal.ΔF. Because the Ad5.βgal.ΔF is deleted in E1, E3 and fiber, there is an additional gene deletion, which should render it very suitable for gene therapy. In addition, the presence of the fiber gene deletion decreases the opportunity to generate replication-competent virus via recombination in the packaging cells. A single Ad vector preparation can be retargeted to any number of different cell types simply by transfecting the cells with the appropriate fiber-expression construct.

EXAMPLE 8

Preparation of Adenoviral Gene Delivery Vectors Containing the Ad37 fiber protein

This example describes construction of packaging cell lines expressing the Ad37 fiber protein, and their use in generating particles of a fiber-deleted Ad vector (such as Ad5.βgal.ΔF) containing this fiber protein. The fiber protein is attached to the viral capsid by binding to the penton base protein through its N-terminus, and the Ad37 fiber was modified in order to make its N-terminal sequence more closely match that of the Ad5 protein to ensure that it would efficiently bind the Ad5 penton base in these vectors.

A. Materials and methods

Cell lines and wild-type adenovirus. Human A549 lung carcinoma epithelial cells and human Chang C conjunctival cells (American Type Culture Collection) were maintained in complete Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. Wild-type Ad19p and Ad37 (ATCC) were propagated in A549 cells and purified by banding on $CsCl_2$ density gradients as previously described (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802). Viral protein concentration was determined by the Bio-Rad Protein Assay, and was used to calculate the number of viral particles based on the known molecular weight of Ad2 virions (1 μg = 4 x 10^9 particles).

B. Construction of the Ad37 fiber expressing cell lines and the recombinant Ad37 knob protein.

1. Construction of an Expression Plasmid for the Ad37 Fiber Protein (pDV80)

The plasmid designated pDV80 (see, SEQ ID No. 42) prepared for expression of the Ad37 fiber protein in mammalian cells, uses the same regulatory elements as the elements in pDV60, pDV67, and pDV69 to express the Ad37 fiber in packaging lines. It was constructed in two steps.

First, the Ad37 fiber open reading frame was amplified from Ad37 genomic DNA using synthetic oligonucleotide primers, L37: 5' TGT CCT GGA TCC AAG *ATG* AAG CGC GCC CGC CCC AGC GAA GAT GAC TTC 3'(SEQ ID NO. 43) and 37FR: 5' AAA CAC GGC GGC CGC TCT TTC ATT CTT G 3' SEQ ID NO. 44). L37 contains nucleotides (underlined) that differ from the Ad37

genomic sequence in order to add a unique *Bam*H1 site (bold) before the start codon (italicized) and to create point mutations that make the N-terminal sequence of the fiber more closely match the N-terminal sequence of the Ad5 fiber protein as follows:

```
Ad37 MSKRLRVEDDFNPVYPY  (SEQ ID No. 45)
      ↓↓↓↓↓↓
      KRARPS            (SEQ ID No. 46)
Ad5  MKRARPSEDTFNPVYPY  (SEQ ID No. 47).
```

37FR also incorporates a unique *Not*1 site (bold). The PCR product was inserted into the *Bam*H1 and *Not*1 sites of pCDNA3.1zeo(+) (Invitrogen) to create pDV78. The correct sequence of the Ad37 fiber protein, including inserted changes, was confirmed by sequencing.

Two point mutations in the fiber gene in the 705 line, S356 to P356 and I362 to T362, were discovered by the sequencing. The mutations are not in the receptor binding domain in Ad37 fiber gene in the 705 cell line. They are buried in the knob trimer interface. To confirm that the these mutations do not affect receptor binding, the Ad37 fiber protein with the correct sequence was recloned, and 293T cells transfected with the virus and subsequently infected with Ad5.GFPΔF to produce Ad37 pseudotyped virus. The results were the same as the results of the experiments with Ad37 pseudotyped virus produced from line 705 (see, Wu *et al.* (2001) *Virology 279*:78-89).

Second, a 1.2 kb *Bam* H1/*Bgl* II fragment containing an adenovirus type 5 tripartite leader was excised from pDV55 (see EXAMPLE 3) and inserted into the *Bam* H1 site of pDV78 to create pDV80 (SEQ ID No 42). Plasmid pDV80 has been deposited in the ATCC under accession number PTA-1147.

2. Construction of the recombinant Ad37 knob protein

Recombinant Ad37 knob protein containing an N-terminal T7•Tag was produced in *E. coli* using the PET expression system (Novagen). Ad37 fiber DNA (GenBank accession number U69132) was PCR amplified from wild-type Ad37 genomic DNA using the following primers (SEQ ID Nos. 48 and 49):

5' <u>GGATCC</u>*ATG*GGATACTTGGTAGCA 3' (*Bam*HI site underlined and

5' GCAA<u>CTCGAG</u>TCATTCTTGGGCAATATAGG 3'(XhoI site underlined).

The PCR reactions were performed at 94°C (denaturation), 55°C (annealing), 72°C (extension, 30 cycles) using *Taq* DNA polymerase (Qiagen). The amplified DNA fragments, which contained residues 172 to 365 of the Ad37 fiber protein with the addition of an N-terminal start codon (italicized), were purified and subcloned into the pCR-TOPO vector using the TA-Cloning Kit (Invitrogen). No replication errors were found by DNA sequencing. Plasmids from cultured transformed colonies were purified and digested with *Bam*HI and *Xho*I. The fragment was inserted into the *Bam*HI and *Xho*I sites of the bacterial expression vector, pET21a (Novagen), and transformed into (DE3)pLYS S expression cells (Invitrogen). Colonies were selected for knob expression by induction with 1 mM IPTG for four hours at 37°C and knob expression was determined by SDS-PAGE. The colony displaying highest knob expression was used for large-scale knob expression and induced with 0.5 mM IPTG at 30°C for four hours.

The recombinant T7•Tagged Ad37 knob protein was purified from sonicated bacteria using the T7•Tag Affinity Purification Kit as recommended by the manufacturer (Novagen). Recovered protein was analyzed for purity by SDS-PAGE followed by Coomassie staining or Western blotting with an HRP-conjugated α-T7•Tag monoclonal antibody as described by the manufacturer (Novagen) or an α-Ad37 fiber rabbit antibody.

3. Preparation of Cell Lines that Express the Ad37 fiber protein

Plasmid pDV80 DNA was purified using the Qiagen method and electroporated into the adenovirus-complementing cell line E1-2a S8 (see Examples herein; see also, Gorziglia *et al.* (1996) *J. Virology 70*:4173-4178; and Von Seggern *et al.* (1998) *J. Gen. Virol. 79*:1461-1468). Stable clones were selected with 600 μg/ml zeocin (Invitrogen).

Clones were expanded and were screened for fiber expression by indirect immunofluorescence (Von Seggern *et al.* (1998) *J.Gen.Virol. 79*:1461-1468) using a rabbit polyclonal antibody directed against the Ad37 fiber (α-Ad37 fiber rabbit antibody) raised by immunizing rabbits with recombinant Ad37 fiber protein. Two clones (lines 705 and 731) that expressed the protein at a uniformly high level were selected.

EXAMPLE 9

Production of Pseudotyped Ad Vector Particles

To generate vector particles equipped ('pseudotyped') with the Ad37 fiber protein, the Ad37 fiber-expressing 705 cells were infected (approximately 1000 particles/cell) with Ad5.βgal.ΔF or with Ad5.GFP.ΔF.

Materials and methods

Ad5.βgal.ΔF

The construction of Ad5.βgal.ΔF is described in Example 2 (it has been deposited on January 15, 1999, with the ATCC as listed above under accession number VR2636; see also, Von Seggern *et al.* (1999) *J. Virol.* *73*:1601-1608; copending U.S. application Serial No. 09/482,682 filed January 14, 2000, and also International PCT application No. PCT/US00/00265, filed January 14, 2000).

Ad5.GFP.ΔF

Ad5.GFP.ΔF was constructed by recombination in bacteria using a modification of the AdEasy System (see, U.S. Patent No. 5,922,576; see, also He *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* *95*:2509-2514; the system is publicly available from the authors and other sources).

First, a fiber-deleted genomic plasmid was constructed by removing the fiber gene from pAdEasy-1 (see, U.S. Patent No. 5,922,576; and He *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* *95*:2509-2514; the AdEasy system and vectors are publicly available from He *et al.* at Johns Hopkins University). Plasmid pAdEasy-1 contains the entire Ad5 genome, except for nucleotides 1-3,533, which encompass the E1 genes, and nucleotides 28,,130-30,820, which encompass the E3 gene.

Plasmid pDV43 (see Example 2; see, also Von Seggern *et al.* (1999) *J. Virol.* *73*:1601-1608) was digested with *Pac*1, the ends blunted by treatment with the large fragment of *E. coli* DNA polymerase and dNTPs, and the product re-ligated to produce plasmd pDV76. The resulting plasmid pDV76 is identical to pDV43 except for loss of the *Pac*1 site and contains the right end of the Ad5 genome with E3 and fiber deletions. A 4.23 kb fragment from PDV76 was amplified using the oligonucleotide primers (SEQ ID Nos. 50 and 51:

5' CGC GCT GAC TCT TAA GGA **CTA GTT** TC 3', including the unique *Spe*1 site in the Ad5 genome (bold); and 5' GCG CTT **AAT TAA** CAT CAT CAA TAA TAT ACC TTA TTT T 3', including a new *Pac*1 site (bold) adjacent to the right Ad5 ITR. Hence the resulting PCR amplified fragment contains nucleotides 27,082 to 35,935 of the Ad5 genome with deletions of nucleotides 28,133 to 32,743 (the E3 and fiber genes), and was used to replace the corresponding *Spe*1/*Pac*1 fragment of pAdEasy 1 (see, U.S. Patent No. 5,922,576) to create pDV77.

Second, *E. coli* strain BJ5183 was electroporated with a mixture of pDV77 and *Pme*1-linearized pAdTrack as described (U.S. Patent No. 5,922,576; He *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*:2509-2514), and DNA was isolated from kanamycin-resistant colonies. The resulting plasmid, pDV83, contains a complete Ad5 genome with E1-, E3-, and fiber-deletions with a CMV-driven GFP reporter gene inserted at the site of the E1 deletion. The full length Ad chromosome was isolated by *Pac*1 digestion, and transfected into the E1- and fiber-complementing 633 cells (Von Seggern *et al.* (2000) *J. Virol. 74*:354-362). The 633 cells were produced by electroporating pDV67 (SEQ ID No. 30, deposited under ATCC accession number PTA-1145) into the E1-2a S8 cells, described above. The recovered virus Ad5.GFP.ΔF was then plaque purified by plating on 633 cells and virus stocks were prepared by freeze-thawing cell pellets.

Ad5-pseudotype particle production

Particles with Ad5 fiber

Ad5-pseudotyped particles were generated by virus growth in 633 cells, which express the wild type Ad5 fiber protein. Viral particles were isolated and purified over CsCl gradients (Von Seggern *et al.* (1999) *J. Virol. 73*:1601-1608; purified by centrifugation on preformed 15-40% CsCl gradients (111,000 x g for three hours at 4°C)). For analysis of viral proteins, ten μg of the purified particles were electrophoresed on 8-16% gradient gels and the protein transferred to nylon membranes. The resulting blot was probed with rabbit polyclonal antibodies raised against recombinant Ad37 fiber or Ad5 fiber or penton base proteins expressed in baculovirus-infected cells.

Particles with Ad37 fiber

Cells from the Ad37 fiber producing cell line 705 were infected at approximately 1000 particles/cell with Ad5.βgal.ΔF or with Ad5.GFP.ΔF. Viral particles were isolated and purified over CsCl gradients. The bands were harvested, dialyzed into storage buffer (10 mM Tris-pH 8.1, 0.9% NaCl, and 10% glycerol), aliquoted and stored at -70°C.

Viral protein analyses

For analysis of viral proteins, 10 μg of purified Ad5.βgal.ΔF particles with no fiber (grown in 293 cells), the Ad5 fiber (grown in 633 cells), or the Ad37 fiber (grown in 705 cells) were electrophoresed by 8-16% polyacrylamide gradient SDS-PAGE and the proteins were transferred to nylon membranes. The blot was then probed with α-Ad37 fiber rabbit antibody. Ad5 fiber and penton base were detected by reprobing the blot with polyclonal antibodies raised against recombinant proteins expressed in baculovirus-infected cells (Wickman *et al.* (1993) *Cell* 2:309-319).

Adenovirus infection and cell binding assays

Adherent Chang C and A549 cells were infected with GFP expressing Ad5 vectors containing the Ad5 fiber (Ad5.GFP.ΔF/5F) or the Ad37 fiber (Ad5.GFP.ΔF/37F) at 10,000 particles per cell for 3 hours at 37°C, 5% CO_2 in DMEM, 10% FCS. Cells were washed twice with saline and then cultured overnight at 37°C, 5% CO_2. The next day, the cells were detached with buffer containing 0.05% (w/v) trypsin and 0.5 mM EDTA (Boehringer Mannheim) for 5 minutes at 37°C. Suspended cells were washed once with PBS and then resuspended in phosphate-buffered saline (PBS), pH 7.4. GFP fluorescence was measured with a FACScan flow cytometer. A threshold established by the fluorescence of uninfected cells was used to distinguish cells expressing GFP. To assess the role of CAR in Ad infection, 10,000 attached cells were pre-incubated with 180 μg/ml RmcB, a function-blocking anti-CAR monoclonal antibody (Hsu *et al.* (1988) *J. Virol.* *62*:1647-1652), in complete DMEM for 1 hour at 4°C. A small volume containing Ad5.GFP.ΔF/5F or Ad5.GFP.ΔF/37F was then added at 10,000 particles per cell. The cells were infected for 3 hours, cultured overnight, harvested, and analyzed for GFP expression. Percent

cells expressing GFP was determined by the percent of cells detected above a threshold set by the fluorescence of uninfected Chang C cells.

To measure adenovirus binding to cells, wild type Ad37 was labeled with ^{125}I using Iodogen (Pierce) according to manufacturer instructions and separated from free ^{125}I by gel filtration as described (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802). Binding of radiolabeled wild type Ad37 on Chang C cells was then quantitated as described (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802). Non-specific binding was determined by incubating cells and labeled Ad37 particles in the presence of 100-fold concentration of unlabeled Ad37. Specific binding was calculated by subtracting the non-specific binding from the total cpm bound. To examine if divalent cations are required for binding, 10 mM ethylenediaminetetraacetic acid (EDTA) or various concentrations of $CaCl_2$, or $MgCl_2$ were added to cells before incubation with labeled virus. To examine if the receptor for Ad37 is a protein, cells were pretreated with 10 μg/ml trypsin (GIBCO), subtilisin (Sigma), proteinase K (Boehringer-Mannheim), and bromelain (Sigman) at 37°C for 1 hour, then washed twice with complete DMEM before adding labeled virus. Cells were >95% viable after protease treatment.

Ad37 binding to conjunctival cells is calcium-dependent. Specific ^{125}I-labeled Ad37 binding to Chang C cells was measured in the presence of 10 mM EDTA and in the presence of varying concentrations of calcium chloride or magnesium chloride. Specific binding was determined by subtracting the nonspecific counts in the presence of 100-fold excess unlabeled virus from the total counts.

Pretreatment of conjunctival cells with proteases inhibits Ad37 binding. Change C cells were pretreated with various proteases for 1 hour before binding ^{125}I-labeled Ad37 to the cells. Nonspecific binding was measured by adding 100-fold unlabeled Ad37 to cells with ^{125}I-labeled Ad37 and subtracting from total counts for specific binding. Percent inhibition represents the difference in specific binding of untreated cells and pretreated cells as a percentage of the specific binding of untreated cells.

Virus overlay protein blot assay (VOPBA)

For VOPBA of human conjunctival membrane proteins probed with Ad37 in the presence of EDTA or calcium chloride, Chang C membrane fractions were separated by 8% SDS-PAGE and transferred to a PDVF membrane. The membrane was subsequently probed with or without whole Ad37 particles, a polyclonal antibody against Ad37 fiber, and finally a horseradish peroxidase conjugated anti-rabbit antibody, in the presence of EDTA or calcium chloride. Transferred Chang C membrane proteins were probed with recombinant Ad37 knob protein, instead of Ad37 knob, in the presence of calcium chloride.

Confluent monolayers of Chang C and A549 cells were detached by scraping, pelleted by centrifugation, and then resuspended in 250 mM sucrose, 20 mM HEPES, pH 7.0, 1 mM EDTA, and 2 μg/ml aprotinin and leupeptin. Cells were transferred into a dounce homogenizer and disrupted with 30 strokes. Organelles and nuclei were pelleted at 500g for 15 min. Plasma membrane fragments were then pelleted from the supernatant of cell lysates at 200,000g for 1 hour and then resuspended in 10 mM Tris•Cl, pH 8.1, 10 μg/ml aprotinin and leupeptin.

Cell membranes of Chang C or A549 cells were incubated (1:1) with a 2% SDS, non-reducing buffer and separated on an 8% polyacrylamide gel without boiling. Membrane proteins were then electroblotted onto a PVDF membrane (Immobilon-P) and blocked in 5% (w/v) milk in PBS, pH 7.4, 0.02% Tween-20 (PBS-T). After blocking, the membrane was incubated with 1 μg/ml wild-type Ad19p or Ad37 in 0.5% (w/v) milk in PBS-T, 1 mM $CaCl_2$, for 1 hour at room temperature. The membrane was then washed once with phosphate-buffered saline, pH 7.4 (PBS), 1 mM $CaCl_2$, and incubated with 1:500 dilution of α-Ad37 fiber rabbit antibody in 0.5% (w/v) milk in PBS-T, 1mM $CaCl_2$, for 30 minutes at room temperature. The membrane was washed again with PBS, 1 mM $CaCl_2$, and incubated with 1:5000 dilution of horseradish peroxidase (HRP) conjugated α-rabbit antibody (Sigma) in 0.5% (w/v) milk in PBS-T, 1 mM $CaCl_2$, for 30 minutes at room temperature. The membrane was washed four times in PBS, 1 mM $CaCl_2$, once with PBS-T, 1 mM $CaCl_2$, and once in 1 mM $CaCl_2$. The blot was developed with enhanced chemiluminescence reagents (Pierce) for 5

minutes and placed onto a piece of Biomax film (Kodak) for 5 seconds to 1 minute. For divalent metal cation experiments, membranes were incubated in the presence of 2 mM EDTA instead of 1 mM $CaCl_2$ in all solutions. To assay fiber knob binding to cell membrane proteins, membrane filters were incubated with 1 μg/ml purified T7-tagged Ad37 knob protein in Tris-buffered saline, 0.1% Tween-20, 1 mM $CaC1_2$, for 1 hour at room temperature. α-Ad37 fiber rabbit antibody and HRP-conjugated anti-rabbit antibody were applied and the membrane was developed with substrate solution as described above.

Results: Comparison of adenovirus infection of human conjunctival and lung epithelial cells with virus particles retargeted with Ad5 or Ad37 fiber proteins

Packaging cell lines producing the Ad37 fiber protein were generated. Since the N-terminal amino acid sequences of the Ad5 and Ad37 fiber proteins differ significantly, and to ensure that the Ad37 fiber would be efficiently incorporated into Ad5 vector particles, several residues in the wild-type Ad37 fiber were mutated to more closely match the Ad5 sequence. Stable cell lines producing this fiber under control of the CMV promoter and the adenovirus type 5 tripartite leader were then generated and screened for fiber expression by indirect immunofluorescence. One clone (line 705), which expressed the Ad37 fiber at a high level, was selected for further study.

Cells from one cell line 633, which expresses the wild-type Ad5 fiber protein, and line 705 were infected with a fiber-deleted Ad5 vector carrying a βgalactosidase reporter gene. The resulting vector particles contained the Ad5 fiber protein (Ad5.βgal.ΔF/5F) and the Ad37 fiber protein (Ad5.βgal.ΔF/37F), respectively. Incorporation of the correct fiber protein into viral particles was verified by Western blotting. Adenoviral vectors containing the GFP reporter gene, Ad5.GFP.ΔF/5F and Ad5.GFP.ΔF/37F, were created in the same fashion.

Infection of a variety of cell types using the retargeted adenovirus particles was examined. As assayed by GFP fluorescence, Ad5.GFP.ΔF/5F exhibited good gene delivery to lung epithelial (A549) and conjunctival cells (Chang C). In contrast, Ad5.GFP.ΔF/37F efficiently delivered GFP to Chang C cells, but exhibited very poor gene delivery to A549 cells. Although CAR is expressed on the surface of A549 cells, as indicated by AD5.GFP.ΔF/5F

infection, Ad5.GFP.ΔF/37F was unable to infect these cells efficiently. This experiment shows that the Ad37 fiber protein can confer preferential infection of human conjunctival cells, but not CAR-expressing human lung epithelial cells.

Hence CAR is not the primary receptor for Ad37. Recent studies reported that expression of CAR on the surface of chinese hamster ovary (CHO) cells did not improve Ad37 binding (Arnberg *et al.* (2000) *J. Virol. 74*:42-48), implying that Ad37 does not use CAR as a primary receptor. In order to verify this on human conjunctival cells, A549 and Chang C cells were pretreated with RmcB (Hsu *et al.* (1988) *J. Virol. 62*:1647-1652), a function-blocking monoclonal antibody against CAR. The RmcB antibody inhibited infection of A549 cells by Ad5.GFP.ΔF/5F, but it had little effect on infection of Chang C cells by Ad5.GFP.ΔF/37F. This indicates that CAR is not the primary receptor for Ad37 on Chang C conjunctival cells.

Ad37 binding to conjunctival cells requires divalent metal cations. It has been proposed (Roelvink *et al.* (1998) *J. Virol. 72*:7909-7915) that a combination of fiber binding to CAR and penton base binding to α_v-integrins allows some adenovirus serotypes to attach to cells. Although α_v-integrin binding to the RGD motif of the adenovirus penton base is of relatively low affinity (Wickman *et al.* (1993) *Cell* 2:309-319), it may nonetheless contribute to viral attachment to the cell surface. Ad37 shows a particularly strong affinity for binding to integrin $\alpha_v\beta_5$ (Mathias *et al.* (1998) *J. Virol. 72*:8669-8675), suggesting that integrin $\alpha_v\beta_5$ might be a primary receptor for Ad37. Binding of the RGD motif by α_v-integrins requires the presence of divalent cations, such as calcium or magnesium (Stuiver *et al.* (1996) *J. Cell Physiol. 168*:521-531). In contrast, no divalent cations were required for binding in the CAR-Ad12 knob complex (Bewley *et al.* (1999) *Science 286*:1579-1583).

To investigate the potential role of α_v-integrins and divalent metal cations in Ad37 receptor binding, ^{125}I-labeled Ad37 binding to Chang C cells was examined in the absence or presence of EDTA. EDTA inhibited Ad37 binding to conjunctival cells but did not alter Ad5 binding. These findings suggest a requirement for divalent metals for Ad37 binding.

The presence of either calcium or magnesium ions helps $\alpha_v\beta_5$ organize in focal contacts (Stuiver *et al.* (1996) *J.Cell Physiol.* *168*:521-531), suggesting that calcium and magnesium aid in integrin $\alpha_v\beta_5$ function. To further test the potential role of integrin $\alpha_v\beta_5$ in Ad37 cell attachment, ^{125}I-labeled Ad37 binding to Chang C cells was measured in the presence of varying concentrations of calcium or magnesium chloride. Magnesium ions had little effect on Ad37 binding to Chang C cells. In contrast, calcium ions dramatically enhanced Ad37 binding to Chang C cells. The optimal concentration of calcium chloride for Ad37 binding was 1 mM, while higher concentrations of calcium actually decreased virus binding to cells. The fact that calcium, but not magnesium, promoted Ad37 attachment is not consistent with integrin $\alpha_v\beta_5$ as the primary receptor for viral attachment to the cells since either metal will support ligand binding to integrin $\alpha_v\beta_5$. Moreover, A549 cells express abundant α_v-integrins (Mathias *et al.* (1998) *J.Virol.* *72*:8669-8675) but were unable to support efficient binding of Ad37.

Wild-type Ad37 particles bind to three conjunctival membrane proteins. Recent studies reported that protease treatment of CHO cells abolished Ad37 binding (Arnberg *et al.* (2000) *J. Virol.* *74*:42-48), implying that Ad37 bound to a protein receptor on CHO cells. Scatchard analysis of Ad37 binding to Chang C cells showed that each cell expresses approximately 24,000 fiber binding sites (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802). To determine if the Ad37 binding site on human conjunctival cells is also a protein, Chang C cells were treated with different proteases prior to measuring binding of ^{125}I-labeled Ad37. Digestion of surface proteins by all four proteases inhibited Ad37 binding to Chang C cells by greater than 50%. This finding showed that Ad37 also binds to a protein receptor on Chang C cells.

Virus overlay protein blot assays (VOPBAs) were used to identify candidate viral protein receptors. This Western blot technique uses intact viral particles in place of antibodies to probe viral-receptors interactions. VOPBA was used herein to identify Chang C membrane proteins that bind to Ad37. In the absence of Ad37 particles, no protein bands were observed, while addition of virus in the absence of calcium revealed binding to a single 45 kDa protein. In

the presence of 1 mM calcium chloride, Ad37 reacted with three proteins with approximate molecular weights of 45, 50 and 60 kDa. The same three proteins were detected using a recombinant Ad37 fiber knob alone, indicating that Ad37-receptor interactions are fiber mediated and do not involve interactions of other capsid proteins such as the penton base. The size of the calcium-independent protein (45 kDa) is very similar to the known molecular weight of CAR. A direct comparison of the Ad37 VOPBA and a CAR Western blot showed that the 45 kDa receptor co-migrates with CAR on SDS-PAGE. Moreover, two other members of subgroup D adenoviruses, Ad9 and AD15, have been shown to bind to CAR (Roelvink *et al.* (1998) *J.Virol.* *72*:7909-7915).

Since CAR does not appear to mediate Ad37 binding on intact Chang C cells, the possibility that the 50 or 60 kDa protein serves this function was tested by examining an adenovirus serotype that does not bind to Chang C cells. Ad19p, a closely related subgroup D adenovirus, binds poorly to Chang C cells (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802) and Ad19p recognition of the Ad37 receptor is therefore unlikely. Ad19p particles bound to the 45 and 60 kDa receptors in the VOPBA, but did not bind to the 50 kDa receptor. Moreover, the 50 kDa receptor is expressed on Chang C cells, but not A549 cells, which only support low levels of Ad37 binding and infection. Taken together, these data indicate that the 50 kDa protein is a primary candidate receptor for Ad37 on human conjunctival cells.

Discussion

The identification of the CAR protein as a major adenovirus receptor does not explain why certain subgroup D members, such as Ad37, preferentially infect ocular cells. A 50 kDa human conjunctival cell membrane protein is identified herein as a primary candidate for the receptor for Ad37. This 50 kDa protein is not present on A549 lung epithelial cells. Ad37 binding to this receptor is calcium-dependent, which is consistent with Ad37 binding and infection experiments. Ad37 also bound to a 60 kDa protein that is present on human conjunctival and lung epithelial cells. It does not, however, appear to be serotype specific. The molecular weights of MHC class I heavy chain, which has been proposed as a receptor for Ad5, and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ intergrins, receptors for

the penton base, are distinct from the 50 or to kDa receptor characterized in this study.

The studies of Ad37-receptor interaction using VOPBAs are consistent with previous studies showing that subgroup D adenoviruses can bind to the extracellular domain of CAR (Roelvink *et al.* (1998) *J.Virol.* *72*:7909-7915). Biochemical and structural studies on knob-CAR interactions indicate that the CAR binding site is located on the AB-loop of the fiber knob. Alignment of the fiber sequences of Ad37 and other adenoviruses reveals that the AB-loop of Ad37 is similar to those of Ad12 and Ad5. Moreover, a phylogenetic tree of adenovirus knobs (Roelvink *et al.* (1998) *J.Virol.* *72*:7909-7915) shows that fiber proteins of subgroup D are similar to those of subgroup C and E, which use CAR as their primary receptor. Ad37 does not, however, appear to effectively use CAR as a primary receptor, as demonstrated by virus binding and infection studies on Chang C conjunctival cells and A549 lung epithelial cells.

It has been reported that Ad37 uses sialic acid as a receptor on chinese hamster ovary (CHO) cells and human lung carcinoma (A549) cells (Arnberg *et al.* ((2000) *J. Virol.* *74*:42-48). Human conjunctival cells were not studied. Human corneal epithelial (HCE) cells were the only ocular cell line studied and Ad37 binds relatively poorly to these cells, compared to binding on A549 cells (Arnberg *et al.* ((2000) *J. Virol.* *74*:42-48). In addition, 8,4 X 10^7 wheat germ agglutinin molecules per cell were required to significantly inhibit Ad37 binding to sialic acid on sialic acid positive CHO cells (Arnberg *et al.* (2000) *J. Virol.* *74*:42-48), three orders of magnitude higher than the number of Ad37 receptors on Chang C conjunctival cells (Huang *et al.* (1999) *J. Virol.* *73*:2798-2802). Clearly, sialic acid is not the only factor responsible for Ad37 binding to the cell surface and its influence on Ad37 tropism is unclear.

The results herein show that Ad37 selects a 50 kDa cellular receptor for binding to conjunctival cells, but it is possible that sialic acid also plays a role in this interaction. The characterization and identification of the Ad37 receptor have therapeutic implications and also explain the different tropism of Ad37. The 50 kDA receptor for Ad37 may also be the receptor for other subgroup D adenoviruses that cause severe cases of EKC, Ad19a and Ad8. Ad19p is a

nonpathogenic variant of Ad19 (Arnberg *et al.* (1998) *Virology 227*:239-244) while Ad19A, along with Ad8 and Ad37, are major causes of EKC. Ad19a and Ad37 have identical fiber proteins (Arnberg *et al.* (1998) *Virology 227*:239-244) and have similar tropism in vivo. Ad8, Ad19a, and Ad37 agglutinate dog and guinea pig erythrocytes more effectively than four other serotypes that are associated with less severe forms of conjunctivitis (Arnberg *et al.* (1998) *Virology 227*:239-244), implying that the receptors of Ad18, Ad19A, and Ad37 have similar characteristics. Hence, this 50 kDa receptor is an attractive drug target against EKC caused by adenoviruses to provide therapeutic intervention of ocular diseases associated with these viruses.

EXAMPLE 10

Targeting of the Ad5 vector to photoreceptor cells

The fiber-deleted adenovirus vector Ad5.GFP.ΔF was propagated in 705 cells, which express a modified Ad37 fiber protein. Viral particles (Ad5.GFP.Δf/37F) were harvested, CsCl-purified and dialized into 0.9% NaCl, 10 mM Tris, pH 8.1, and 10% glycerol. Two to three μl of the resulting solution, containing approximately 1 x 10^9 particles/μl was injected into the vitreous chamber of a mouse eye. Seven days post-injection, eyes were harvested, fixed with paraformaldehyde and cryo-sectioned. Sections were stained with an anti-rhodopsin antibody to identify photoreceptor cells and with DAPI to show all cell nuclei. The resulting sections showed red anti-rhodopsin staining in the photoreceptors, blue DAPI-stained nuclei, and green GFP staining in any transduced cells. The results revealed substantially exclusive transduction of photoreceptors. Co-localization of rhodopsin staining and GFP expression indicated selective transduction of photoreceptor cells.

As a control, contralateral eyes were injected with a stock of the fiber-deleted vector AD5.βgal.ΔF grown in the same Ad37 fiber-expressing cells. Since this virus (Ad5.βgal.ΔF/37F) produces βgal rather than GFP, the green staining is absent from the photoreceptors.

Additional experiments using the AD37 fiber for targeting to the photoreceptor cells have been performed. Subretinal and intravitreal injection have been used in mouse models and the results demonstrate targeting to the

photoreceptors. As with intravitreally injected eyes, the major cell type infectd via subretinal administration was the photoreceptor.

As noted, Ad5.GFP.ΔF /37F infected Chang C cells efficiently, but A549 cells poorly. Ad37 fiber protein confers preferential infection on human conjunctival cells, but not CAR-expressing human lung epithelial cells. Binding to conjunctival cells requires divalent cations.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule, comprising:

adenovirus inverted terminal repeat sequences; an adenovirus packaging signal operatively linked thereto; and a photoreceptor-specific promoter.

2. The isolated nucleic acid molecule of claim 1, further comprising a nucleic acid encoding a therapeutic product operatively linked to the promoter.

3. The isolated nucleic acid molecule of claim 1, wherein the promoter is a rhodopsin promoter.

4. The nucleic acid molecule of claim 1, wherein the adenovirus genome does not encode a functional fiber protein such that packaging the nucleic acid requires complementation in a packaging cell.

5. A recombinant adenovirus vector, comprising the nucleic acid molecule of any of claims 1-4 packaged therein.

6. A recombinant adenovirus vector of claim 5, wherein inverted terminal repeat sequences (ITR) and a packaging signal are derived from adenovirus type 2 or adenovirus type 5.

7. A recombinant adenovirus vector of claim 5, wherein the virus comprises a fiber protein.

8. A recombinant adenovirus vector of claim 7, wherein the fiber protein selectively binds to photoreceptors in the eye of a mammal.

9. A recombinant adenovirus vector of claim 7, wherein the fiber is a chimera composed of N-terminal sequences from adenovirus type 2 or type 5, and a sufficient portion of an adenovirus serotype D fiber for selective binding to photoreceptors in the eye of a mammal.

10. A method for targeted delivery of a gene product to the eye of a mammal, comprising:

administering a recombinant adenovirus virus that comprises heterologous DNA encoding the gene product or resulting in expression of the gene product, wherein the recombinant virus comprises a fiber protein that specifically or selectively binds to receptors that are expressed on cells in the eye.

11. The method of claim 10, wherein the cells are photoreceptors.

12. The method of claim 10, wherein administration is effected by intraocular delivery.

13. The method of claim 10, wherein administration is effected by a method selected from subretinal injection, intravenous administration, periorbital administration, and intravitreal administration.

14. The method of claim 10, wherein the recombinant virus comprises a fiber protein from an adenovirus type D serotype.

15. The method of any of claims 10-14, wherein the fiber protein is an adenovirus type 37.

16. The method of any of claims 10-14, wherein the fiber is a chimeric protein containing a sufficient portion of the N-terminus of an adenovirus type 2 or type 5 fiber protein for interaction with an adenovirus type 2 or type 5 penton, and a sufficient portion of an adenovirus serotype D knob portion of the fiber for selective binding to photoreceptors in the eye of a mammal.

17. The method of any of claims 10-16, wherein the recombinant virus is an adenovirus type D serotype.

18. The method of any of claims 10-17, wherein the encapsulated nucleic acid comprises a photoreceptor-specific promoter operatively linked to a nucleic acid comprising the therapeutic product.

19. The method of claim 18, wherein the therapeutic product is selected from the group consisting of a trophic factor, an anti-apoptotic factor, a gene encoding a rhodopsin protein, a wild-type Stargardt disease gene (STDG1), an anti-cancer agent and a protein that regulates expression of a photoreceptor-specific gene product.

20. The method of any of claims 10-19, wherein delivery is effected for treatment of an ocular disease.

21. The method of claim 20, wherein the disorder is a retinal degenerative disease.

22. The method of claim 20, wherein the disease is retinitis pigmentosa, Stargardt's disease, diabetic retinopathies, retinal vascularization, or retinoblastoma.

23. The method of any of claims 10-22, wherein the mammal is a human.

24. The method of any of claims 10-22, wherein the viral nucleic acid comprises:

an adenovirus inverted terminal repeat (ITR) sequences; and

an adenovirus packaging signal operatively linked thereto.

25. The method of claim 24, wherein the ITRs and packaging signal are derived from an adenovirus serotype B or C.

26. The method of claim 24, wherein the ITRs and packaging signal are derived from an adenovirus type 2 or 5.

27. The method of claim 24, wherein the viral nucleic acid further comprises a photoreceptor-specific promoter.

28. A method of targeted gene therapy, comprising:

administering a recombinant viral vector that comprises an adenovirus type 37 fiber protein or portion thereof, whereby the vector selectively transduces photoreceptors and delivers a gene product encoded by the recombinant viral vector; wherein the portion is sufficient for selective binding to photoreceptors.

29. The method of claim 28, wherein the vector is administered into the eye.

30. The method of claim 28, wherein the vector is administered to the vitreous cavity of the eye.

31. The method of claim 28, wherein administration is effected by subretinal injection, intravenous administration, periorbital administration or intravitreal administration.

32. The method of any of claims 10-31, wherein at least about 10^7 plaque forming units of virus are administered.

33. The method of any of claims 10-31, wherein about 1 plaque forming unit to about 10^{14} plaque forming units of virus are administered.

SEQUENCE LISTING

<110> VON SEGGERN, DANIEL
NEMEROW, GLEN R.
FRIEDLANDER, MARTIN

<120> VECTORS FOR OCULAR TRANSDUCTION AND USE THEREFOR FOR GENETIC THERAPY

<130> 756.1PCT/NOV0205P

<140>
<141> 2001-05-01

<150> 09/562,934
<151> 2000-05-01

<160> 51

<170> PatentIn Ver. 2.1

<210> 1
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 1
cggtacacag aattcaggag acacaactcc 30

<210> 2
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 2
gcctggatcc gggaagttac gtaacgtggg aaaac 35

<210> 3
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker

<400> 3
cgcggatccg cg 12

<210> 4
<211> 8710
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: plasmid

<400> 4
cacctaaatt gtaagcgtta atattttgtt aaaattcgcg ttaaattttt gttaaatcag 60
ctcatttttt aaccaatagg ccgaaatcgg caaaatccct tataaatcaa aagaatagac 120
cgagataggg ttgagtgttg ttccagtttg gaacaagagt ccactattaa agaacgtgga 180
ctccaacgtc aaagggcgaa aaaccgtcta tcagggcgat ggcccactac gtgaaccatc 240
accctaatca agttttttgg ggtcgaggtg ccgtaaagca ctaaatcgga accctaaagg 300
gagcccccga tttagagctt gacggggaaa gccggcgaac gtggcgagaa aggaagggaa 360
gaaagcgaaa ggagcgggcg ctagggcgct ggcaagtgta gcggtcacgc tgcgcgtaac 420
caccacaccc gccgcgctta atgcgccgct acagggcgcg tcccattcgc cattcaggct 480
gcgcaactgt tgggaagggc gatcggtgcg ggcctcttcg ctattacgcc agctggcgaa 540
agggggatgt gctgcaaggc gattaagttg ggtaacgcca gggttttccc agtcacgacg 600
ttgtaaaacg acggccagtg aattgtaata cgactcacta tagggcgaat tgggtaccgg 660
gccccccctc gaggtcgacg gtatcgataa gcttgatatc gaattcagga gacacaactc 720
caagtgcata ctctatgtca ttttcatggg actggtctgg ccacaactac attaatgaaa 780
tatttgccac atcctcttac actttttcat acattgccca agaataaaga atcgtttgtg 840
ttatgtttca acgtgtttat ttttcaattg cagaaaattt caagtcattt ttcattcagt 900
agtatagccc caccaccaca tagcttatac agatcaccgt accttaatca aactcacaga 960
accctagtat tcaacctgcc acctccctcc caacacacag agtacacagt cctttctccc 1020
cggctggcct taaaaagcat catatcatgg gtaacagaca tattcttagg tgttatattc 1080
cacacggttt cctgtcgagc caaacgctca tcagtgatat taataaactc cccgggcagc 1140
tcacttaagt tcatgtcgct gtccagctgc tgagccacag gctgctgtcc aacttgcggt 1200
tgcttaacgg gcggcgaagg agaagtccac gcctacatgg gggtagagtc ataatcgtgc 1260
atcaggatag ggcggtggtg ctgcagcagc gcgcgaataa actgctgccg ccgccgctcc 1320
gtcctgcagg aatacaacat ggcagtggtc tcctcagcga tgattcgcac cgcccgcagc 1380
ataaggcgcc ttgtcctccg ggcacagcag cgcaccctga tctcacttaa atcagcacag 1440
taactgcagc acagcaccac aatattgttc aaaatcccac agtgcaaggc gctgtatcca 1500
aagctcatgg cggggaccac agaacccacg tggccatcat accacaagcg caggtagatt 1560
aagtggcgac ccctcataaa cacgctggac ataaacatta cctcttttgg catgttgtaa 1620
ttcaccacct cccggtacca tataaacctc tgattaaaca tggcgccatc caccaccatc 1680
ctaaaccagc tggccaaaac ctgcccgccg gctatacact gcagggaacc gggactggaa 1740
caatgacagt ggagagccca ggactcgtaa ccatggatca tcatgctcgt catgatatca 1800
atgttggcac aacacaggca cacgtgcata cacttcctca ggattacaag ctcctcccgc 1860
gttagaacca tatcccaggg aacaacccat tcctgaatca gcgtaaatcc cacactgcag 1920
ggaagacctc gcacgtaact cacgttgtgc attgtcaaag tgttacattc gggcagcagc 1980
ggatgatcct ccagtatggt agcgcgggtt tctgtctcaa aaggaggtag acgatcccta 2040
ctgtacggag tgcgccgaga caaccgagat cgtgttggtc gtagtgtcat gccaaatgga 2100
acgccggacg tagtcatatt tcctgaagca aaaccaggtg cgggcgtgac aaacagatct 2160
gcgtctccgg tctcgccgct tagatcgctc tgtgtagtag ttgtagtata tccactctct 2220
caaagcatcc aggcgccccc tggcttcggg ttctatgtaa actccttcat gcgccgctgc 2280
cctgataaca tccaccaccg cagaataagc cacacccagc caacctacac attcgttctg 2340
cgagtcacac acgggaggag cgggaagagc tggaagaacc atgttttttt ttttattcca 2400
aaagattatc caaaacctca aaatgaagat ctattaagtg aacgcgctcc cctccggtgg 2460
cgtggtcaaa ctctacagcc aaagaacaga taatggcatt tgtaagatgt tgcacaatgg 2520
cttccaaaag gcaaacggcc ctcacgtcca agtggacgta aaggctaaac ccttcagggt 2580
gaatctcctc tataaacatt ccagcacctt caaccatgcc caaataattc tcatctcgcc 2640
accttctcaa tatatctcta agcaaatccc gaatattaag tccggccatt gtaaaaatct 2700
gctccagagc gccctccacc ttcagcctca agcagcgaat catgattgca aaaattcagg 2760
ttcctcacag acctgtataa gattcaaaag cggaacatta acaaaaatac cgcgatcccg 2820
taggtccctt cgcagggcca gctgaacata atcgtgcagg tctgcacgga ccagcgcggc 2880
cacttccccg ccaggaacct tgacaaaaga acccacactg attatgacac gcatactcgg 2940
agctatgcta accagcgtag ccccgatgta agctttgttg catgggcggc gatataaaat 3000
gcaaggtgct gctcaaaaaa tcaggcaaag cctcgcgcaa aaaagaaagc acatcgtagt 3060
catgctcatg cagataaagg caggtaagct ccggaaccac cacagaaaaa gacaccattt 3120
ttctctcaaa catgtctgcg ggtttctgca taaacacaaa ataaaataac aaaaaaacat 3180
ttaaacatta gaagcctgtc ttacaacagg aaaaacaacc cttataagca taagacggac 3240
tacggccatg ccggcgtgac cgtaaaaaaa ctggtcaccg tgattaaaaa gcaccaccga 3300
cagctcctcg gtcatgtccg gagtcataat gtaagactcg gtaaacacat caggttgatt 3360
catcggtcag tgctaaaaag cgaccgaaat agcccggggg aatacatacc cgcaggcgta 3420
gagacaacat tacagccccc ataggaggta taacaaaatt aataggagag aaaaacacat 3480
aaacacctga aaaaccctcc tgcctaggca aaatagcacc ctcccgctcc agaacaacat 3540

acagcgcttc acagcggcag cctaacagtc agccttacca gtaaaaaaga aaacctatta 3600
aaaaaacacc actcgacacg gcaccagctc aatcagtcac agtgtaaaaa agggccaagt 3660
gcagagcgag tatatatagg actaaaaaat gacgtaacgg ttaaagtcca caaaaaacac 3720
ccagaaaacc gcacgcgaac ctacgcccag aaacgaaagc caaaaaaccc acaacttcct 3780
caaatcgtca cttccgtttt cccacgttac gtaacttccc ggatccgcgg cattcacagt 3840
tctccgcaag aattgattgg ctccaattct tggagtggtg aatccgttag cgaggtgccg 3900
ccggcttcca ttcaggtcga ggtggcccgg ctccatgcac cgcgacgcaa cgcggggagg 3960
cagacaaggt atagggcggc gcctacaatc catgccaacc cgttccatgt gctcgccgag 4020
gcggcataaa tcgccgtgac gatcagcggt ccagtgatcg aagttaggct ggtaagagcc 4080
gcgagcgatc cttgaagctg tccctgatgg tcgtcatcta cctgcctgga cagcatggcc 4140
tgcaacgcgg gcatcccgat gccgccggaa gcgagaagaa tcataatggg gaaggccatc 4200
cagcctcgcg tcgcgaacgc cagcaagacg tagcccagcg cgtcggccgc catgccctgc 4260
ttcatccccg tggccgttg ctcgcgtttg ctggcggtgt ccccggaaga aatatatttg 4320
catgtcttta gttctatgat gacacaaacc ccgcccagcg tcttgtcatt ggcgaattcg 4380
aacacgcaga tgcagtcggg gcggcgcggt cccaggtcca cttcgcatat taaggtgacg 4440
cgtgtggcct cgaacaccga gcgaccctgc agcgacccgc ttaacagcgt caacagcgtg 4500
ccgcagatcc cgggcaatga gatatgaaaa agcctgaact caccgcgacg tctgtcgaga 4560
agtttctgat cgaaaagttc gacagcgtct ccgacctgat gcagctctcg gagggcgaag 4620
aatctcgtgc tttcagcttc gatgtaggag ggcgtggata tgtcctgcgg gtaaatagct 4680
gcgccgatgg tttctacaaa gatcgttatg tttatcggca ctttgcatcg gccgcgctcc 4740
cgattccgga agtgcttgac attggggaat tcagcgagag cctgacctat tgcatctccc 4800
gccgtgcaca gggtgtcacg ttgcaagacc tgcctgaaac cgaactgccc gctgttctgc 4860
agccggtcgc ggaggccatg gatgcgatcg ctgcggccga tcttagccag acgagcgggt 4920
tcggcccatt cggaccgcaa ggaatcggtc aatacactac atggcgtgat ttcatatgcg 4980
cgattgctga tccccatgtg tatcactggc aaactgtgat ggacgacacc gtcagtgcgt 5040
ccgtcgcgca ggctctcgat gagctgatgc tttgggccga ggactgcccc gaagtccggc 5100
acctcgtgca cgcggatttc ggctccaaca atgtcctgac ggacaatggc cgcataacag 5160
cggtcattga ctggagcgag gcgatgttcg gggattccca atacgaggtc gccaacatct 5220
tcttctggag gccgtggttg gcttgtatgg agcagcagac gcgctacttc gagcggaggc 5280
atccggagct tgcaggatcg ccgcggctcc gggcgtatat gctccgcatt ggtcttgacc 5340
aactctatca gagcttggtt gacggcaatt tcgatgatgc agcttgggcg cagggtcgat 5400
gcgacgcaat cgtccgatcc ggagccggga ctgtcgggcg tacacaaatc gcccgcagaa 5460
gcgcggccgt ctggaccgat ggctgtgtag aagtactcgc cgatagtgga aaccgacgcc 5520
ccagcactcg tccgagggca aaggaatagg ggagatgggg gaggctaact gaaacacgga 5580
aggagacaat accggaagga acccgcgcta tgacggcaat aaaaagacag aataaaacgc 5640
acgggtgttg ggtcgtttgt tcataaacgc ggggttcggt cccagggctg gcactctgtc 5700
gataccccac cgagacccca ttggggccaa tacgcccgcg tttcttcctt ttccccaccc 5760
caccccccaa gttcgggtga aggcccaggg ctcgcagcca acgtcggggc ggcaggccct 5820
gccatagcca ctggccccgt gggttaggga cggggtcccc catggggaat ggtttatggt 5880
tcgtgggggt tattattttg ggcgttgcgt ggggtctggt ccacgactgg actgagcaga 5940
cagacccatg gtttttggat ggcctgggca tggaccgcat gtactggcgc gacacgaaca 6000
ccgggcgtct gtggctgcca aacacccccg acccccaaaa accaccgcgc ggatttctgg 6060
cgcccagtgc cgtcgaccgg tcatggctgc gccccgacac ccgccaacac ccgctgacgc 6120
gccctgacgg gcttgtctgc tcccggcatc cgcttacaga caagctgtga ccgtctccgg 6180
gagctgcatg tgtcagaggt tttcaccgtc atcaccgaaa cgcgcgaggc agccggatca 6240
taatcagcca taccacattt gtagaggttt tacttgcttt aaaaaacctc ccacacctccc 6300
cctgaacctg aaacataaaa tgaatgcaat tgttgttgtt aacttgttta ttgcagctta 6360
taatggttac aaataaagca atagcatcac aaatttcaca aataaagcat ttttttcact 6420
gcattctagt tgtggtttgt ccaaactcat caatgtatct tatcatgtct ggatccacta 6480
gttctagagc ggccgccacc gcggtggagc tccagctttt gttcccttta gtgagggtta 6540
atttcgagct tggcgtaatc atggtcatag ctgtttcctg tgtgaaattg ttatccgctc 6600
acaattccac acaacatacg agccggaagc ataaagtgta aagcctgggg tgcctaatga 6660
gtgagctaac tcacattaat tgcgttgcgc tcactgcccg ctttccagtc gggaaacctg 6720
tcgtgccagc tgcattaatg aatcggccaa cgcgcgggga gaggcggttt gcgtattggg 6780
cgctcttccg cttcctcgct cactgactcg ctgcgctcgg tcgttcggct gcggcgagcg 6840
gtatcagctc actcaaaggc ggtaatacgg ttatccacag aatcagggga taacgcagga 6900
aagaacatgt gagcaaaagg ccagcaaaag gccaggaacc gtaaaaaggc cgcgttgctg 6960
gcgtttttcc ataggctccg cccccctgac gagcatcaca aaaatcgacg ctcaagtcag 7020
aggtggcgaa acccgacagg actataaaga taccaggcgt ttccccctgg aagctccctc 7080
gtgcgctctc ctgttccgac cctgccgctt accggatacc tgtccgcctt tctcccttcg 7140
ggaagcgtgg cgctttctca tagctcacgc tgtaggtatc tcagttcggt gtaggtcgtt 7200
cgctccaagc tgggctgtgt gcacgaaccc cccgttcagc ccgaccgctg cgccttatcc 7260

ggtaactatc gtcttgagtc caacccggta agacacgact tatcgccact ggcagcagcc 7320
actggtaaca ggattagcag agcgaggtat gtaggcggtg ctacagagtt cttgaagtgg 7380
tggcctaact acggctacac tagaaggaca gtatttggta tctgcgctct gctgaagcca 7440
gttaccttcg gaaaaagagt tggtagctct tgatccggca aacaaaccac cgctggtagc 7500
ggtggttttt ttgtttgcaa gcagcagatt acgcgcagaa aaaaaggatc tcaagaagat 7560
cctttgatct tttctacggg gtctgacgct cagtggaacg aaaactcacg ttaagggatt 7620
ttggtcatga gattatcaaa aaggatcttc acctagatcc ttttaaatta aaaatgaagt 7680
tttaaatcaa tctaaagtat atatgagtaa acttggtctg acagttacca atgcttaatc 7740
agtgaggcac ctatctcagc gatctgtcta tttcgttcat ccatagttgc ctgactcccc 7800
gtcgtgtaga taactacgat acgggagggc ttaccatctg gccccagtgc tgcaatgata 7860
ccgcgagacc cacgctcacc ggctccagat ttatcagcaa taaaccagcc agccggaagg 7920
gccgagcgca gaagtggtcc tgcaacttta tccgcctcca tccagtctat taattgttgc 7980
cgggaagcta gagtaagtag ttcgccagtt aatagtttgc gcaacgttgt tgccattgct 8040
acaggcatcg tggtgtcacg ctcgtcgttt ggtatggctt cattcagctc cggttcccaa 8100
cgatcaaggc gagttacatg atcccccatg ttgtgcaaaa aagcggttag ctccttcggt 8160
cctccgatcg ttgtcagaag taagttggcc gcagtgttat cactcatggt tatggcagca 8220
ctgcataatt ctcttactgt catgccatcc gtaagatgct tttctgtgac tggtgagtac 8280
tcaaccaagt cattctgaga atagtgtatg cggcgaccga gttgctcttg cccggcgtca 8340
atacgggata ataccgcgcc acatagcaga actttaaaag tgctcatcat tggaaaacgt 8400
tcttcggggc gaaaactctc aaggatctta ccgctgttga gatccagttc gatgtaaccc 8460
actcgtgcac ccaactgatc ttcagcatct tttactttca ccagcgtttc tgggtgagca 8520
aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg cgacacggaa atgttgaata 8580
ctcatactct tcctttttca atattattga agcatttatc agggttattg tctcatgagc 8640
ggatacatat ttgaatgtat ttagaaaaat aaacaaatag gggttccgcg cacatttccc 8700
cgaaaagtgc 8710

<210> 5
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 5
atgggatcca agatgaagcg cgcaagaccg 30

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 6
cataacgcgg ccgcttcttt attcttgggc 30

<210> 7
<211> 7148
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid

<400> 7
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180

ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc 900
gagctcggat ccaagatgaa gcgcgcaaga ccgtctgaag ataccttcaa ccccgtgtat 960
ccatatgaca cggaaaccgg tcctccaact gtgccttttc ttactcctcc ctttgtatcc 1020
cccaatgggt ttcaagagag tcccccgggg gtactctctt tgcgcctatc cgaacctcta 1080
gttacctcca atggcatgct tgcgctcaaa atgggcaacg gcctctctct ggacgaggcc 1140
ggcaacctta cctcccaaaa tgtaaccact gtgagcccac ctctcaaaaa aaccaagtca 1200
aacataaacc tggaaatatc tgcacccctc acagttacct cagaagccct aactgtggct 1260
gccgccgcac ctctaatggt cgcgggcaac acactcacca tgcaatcaca ggccccgcta 1320
accgtgcacg actccaaact tagcattgcc acccaaggac ccctcacagt gtcagaagga 1380
aagctagccc tgcaaacatc aggcccccctc accaccaccg atagcagtac ccttactatc 1440
actgcctcac cccctctaac tactgccact ggtagcttgg gcattgactt gaaagagccc 1500
atttatacac aaaatggaaa actaggacta aagtacgggg ctcctttgca tgtaacagac 1560
gacctaaaca ctttgaccgt agcaactggt ccaggtgtga ctattaataa tacttccttg 1620
caaactaaag ttactggagc cttgggtttt gattcacaag gcaatatgca acttaatgta 1680
gcaggaggac taaggattga ttctcaaaac agacgcctta tacttgatgt tagttatccg 1740
tttgatgctc aaaaccaact aaatctaaga ctaggacagg gccctctttt tataaactca 1800
gcccacaact tggatattaa ctacaacaaa ggcctttact tgtttacagc ttcaaacaat 1860
tccaaaaagc ttgaggttaa cctaagcact gccaaggggt tgatgtttga cgctacagcc 1920
atagccatta atgcaggaga tgggcttgaa tttggttcac ctaatgcacc aaacacaaat 1980
cccctcaaaa caaaaattgg ccatggccta gaatttgatt caaacaaggc tatggttcct 2040
aaactaggaa ctggccttag ttttgacagc acaggtgcca ttacagtagg aaacaaaaat 2100
aatgataagc taactttgtg gaccacacca gctccatctc ctaactgtag actaaatgca 2160
gagaaagatg ctaaactcac tttggtctta acaaaatgtg gcagtcaaat acttgctaca 2220
gtttcagttt tggctgttaa aggcagtttg gctccaatat ctggaacagt tcaaagtgct 2280
catcttatta taagatttga cgaaaatgga gtgctactaa acaattcctt cctggaccca 2340
gaatattgga actttagaaa tggagatctt actgaaggca cagcctatac aaacgctgtt 2400
ggatttatgc ctaacctatc agcttatcca aaatctcacg gtaaaactgc caaaagtaac 2460
attgtcagtc aagtttactt aaacggagac aaaactaaac ctgtaacact aaccattaca 2520
ctaaacggta cacaggaaac aggagacaca actccaagtg catactctat gtcattttca 2580
tgggactggt ctggccacaa ctacattaat gaaatatttg ccacatcctc ttacactttt 2640
tcatacattg cccaagaata aagaagcggc cgctcgagca tgcatctaga gggccctatt 2700
ctatagtgtc acctaaatgc tagagctcgc tgatcagcct cgactgtgcc ttctagttgc 2760
cagccatctg ttgtttgccc ctcccccgtg ccttccttga ccctggaagg tgccactccc 2820
actgtccttt cctaataaaa tgaggaaatt gcatcgcatt gtctgagtag gtgtcattct 2880
attctggggg gtggggtggg gcaggacagc aagggggagg attgggaaga caatagcagg 2940
catgctgggg atgcggtggg ctctatggct tctgaggcgg aaagaaccag ctggggctct 3000
agggggtatc cccacgcgcc ctgtagcggc gcattaagcg cggcgggtgt ggtggttacg 3060
cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg ctcctttcgc tttcttccct 3120
tcctttctcg ccacgttcgc cggctttccc cgtcaagctc taaatcgggg catcccttta 3180
gggttccgat ttagtgcttt acggcacctc gaccccaaaa aacttgatta gggtgatggt 3240
tcacgtagtg ggccatcgcc ctgatagacg gtttttcgcc ctttgacgtt ggagtccacg 3300
ttctttaata gtggactctt gttccaaact ggaacaacac tcaaccctat ctcggtctat 3360
tcttttgatt tataagggat tttggggatt tcggcctatt ggttaaaaaa tgagctgatt 3420
taacaaaaat ttaacgcgaa ttaattctgt ggaatgtgtg tcagttaggg tgtggaaagt 3480
ccccaggctc cccaggcagg cagaagtatg caaagcatgc atctcaatta gtcagcaacc 3540
aggtgtggaa agtccccagg ctccccagca ggcagaagta tgcaaagcat gcatctcaat 3600
tagtcagcaa ccatagtccc gcccctaact ccgcccatcc cgcccctaac tccgcccagt 3660
tccgcccatt ctccgcccca tggctgacta atttttttta tttatgcaga ggccgaggcc 3720
gcctctgcct ctgagctatt ccagaagtag tgaggaggct tttttggagg cctaggcttt 3780
tgcaaaaagc tcccgggagc ttgtatatcc attttcggat ctgatcaaga gacaggatga 3840
ggatcgtttc gcatgattga acaagatgga ttgcacgcag gttctccggc cgcttgggtg 3900

```
gagaggctat tcggctatga ctgggcacaa cagacaatcg gctgctctga tgccgccgtg 3960
ttccggctgt cagcgcaggg gcgcccggtt ctttttgtca agaccgacct gtccggtgcc 4020
ctgaatgaac tgcaggacga ggcagcgcgg ctatcgtggc tggccacgac gggcgttcct 4080
tgcgcagctg tgctcgacgt tgtcactgaa gcgggaaggg actggctgct attgggcgaa 4140
gtgccggggc aggatctcct gtcatctcac cttgctcctg ccgagaaagt atccatcatg 4200
gctgatgcaa tgcggcggct gcatacgctt gatccggcta cctgcccatt cgaccaccaa 4260
gcgaaacatc gcatcgagcg agcacgtact cggatggaag ccggtcttgt cgatcaggat 4320
gatctggacg aagagcatca ggggctcgcg ccagccgaac tgttcgccag gctcaaggcg 4380
cgcatgcccg acggcgagga tctcgtcgtg acccatggcg atgcctgctt gccgaatatc 4440
atggtggaaa atggccgctt ttctggattc atcgactgtg gccggctggg tgtggcggac 4500
cgctatcagg acatagcgtt ggctacccgt gatattgctg aagagcttgg cggcgaatgg 4560
gctgaccgct tcctcgtgct ttacggtatc gccgctcccg attcgcagcg catcgccttc 4620
tatcgccttc ttgacgagtt cttctgagcg ggactctggg gttcgaaatg accgaccaag 4680
cgacgcccaa cctgccatca cgagatttcg attccaccgc cgccttctat gaaaggttgg 4740
gcttcggaat cgttttccgg gacgccggct ggatgatcct ccagcgcggg gatctcatgc 4800
tggagttctt cgcccacccc aacttgttta ttgcagctta taatggttac aaataaagca 4860
atagcatcac aaatttcaca aataaagcat ttttttcact gcattctagt tgtggtttgt 4920
ccaaactcat caatgtatct tatcatgtct gtataccgtc gacctctagc tagagcttgg 4980
cgtaatcatg gtcatagctg tttcctgtgt gaaattgtta tccgctcaca attccacaca 5040
acatacgagc cggaagcata aagtgtaaag cctggggtgc ctaatgagtg agctaactca 5100
cattaattgc gttgcgctca ctgcccgctt tccagtcggg aaacctgtcg tgccagctgc 5160
attaatgaat cggccaacgc gcggggagag gcggtttgcg tattgggcgc tcttccgctt 5220
cctcgctcac tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta tcagctcact 5280
caaaggcggt aatacggtta tccacagaat caggggataa cgcaggaaag aacatgtgag 5340
caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg tttttccata 5400
ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg tggcgaaacc 5460
cgacaggact ataaagatac caggcgtttc cccctggaag ctccctcgtg cgctctcctg 5520
ttccgaccct gccgcttacc ggatacctgt ccgcctttct cccttcggga agcgtggcgc 5580
tttctcaatg ctcacgctgt aggtatctca gttcggtgta ggtcgttcgc tccaagctgg 5640
gctgtgtgca cgaacccccc gttcagcccg accgctgcgc cttatccggt aactatcgtc 5700
ttgagtccaa cccggtaaga cacgacttat cgccactggc agcagccact ggtaacagga 5760
ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtggtgg cctaactacg 5820
gctacactag aaggacagta tttggtatct gcgctctgct gaagccagtt accttcggaa 5880
aaagagttgg tagctcttga tccggcaaac aaaccaccgc tggtagcggt ggtttttttg 5940
tttgcaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct ttgatctttt 6000
ctacggggtc tgacgctcag tggaacgaaa actcacgtta agggattttg gtcatgagat 6060
tatcaaaaag gatcttcacc tagatccttt taaattaaaa atgaagtttt aaatcaatct 6120
aaagtatata tgagtaaact tggtctgaca gttaccaatg cttaatcagt gaggcaccta 6180
tctcagcgat ctgtctattt cgttcatcca tagttgcctg actccccgtc gtgtagataa 6240
ctacgatacg ggagggctta ccatctggcc ccagtgctgc aatgataccg cgagacccac 6300
gctcaccggc tccagattta tcagcaataa accagccagc cggaagggcc gagcgcagaa 6360
gtggtcctgc aactttatcc gcctccatcc agtctattaa ttgttgccgg gaagctagag 6420
taagtagttc gccagttaat agtttgcgca acgttgttgc cattgctaca ggcatcgtgg 6480
tgtcacgctc gtcgtttggt atggcttcat tcagctccgg ttcccaacga tcaaggcgag 6540
ttacatgatc ccccatgttg tgcaaaaaag cggttagctc cttcggtcct ccgatcgttg 6600
tcagaagtaa gttggccgca gtgttatcac tcatggttat ggcagcactg cataattctc 6660
ttactgtcat gccatccgta agatgctttt ctgtgactgg tgagtactca accaagtcat 6720
tctgagaata gtgtatgcgg cgaccgagtt gctcttgccc ggcgtcaata cgggataata 6780
ccgcgccaca tagcagaact ttaaaagtgc tcatcattgg aaaacgttct tcggggcgaa 6840
aactctcaag gatcttaccg ctgttgagat ccagttcgat gtaacccact cgtgcaccca 6900
actgatcttc agcatctttt actttcacca gcgtttctgg gtgagcaaaa acaggaaggc 6960
aaaatgccgc aaaaaaggga ataagggcga cacggaaatg ttgaatactc atactcttcc 7020
tttttcaata ttattgaagc atttatcagg gttattgtct catgagcgga tacatatttg 7080
aatgtattta gaaaaataaa caaatagggg ttccgcgcac atttccccga aaagtgccac 7140
ctgacgtc                                                          7148
```

<210> 8
<211> 7469
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid

<400> 8
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc 900
gagctcggat ctgaattcga gctcgctgtt gggctcgcgg ttgaggacaa actcttcgcg 960
gtctttccag tactcttgga tcggaaaccc gtcggcctcc gaacggtact ccgccaccga 1020
gggacctgag cgagtccgca tcgaccggat cggaaaacct ctcgagaaag gcgtctaacc 1080
agtcacagtc gcaaggtagg ctgagcaccg tggcgggcgg cagcgggtgg cggtcggggt 1140
tgtttctggc ggaggtgctg ctgatgatgt aattaaagta ggcggtcttg agacggcgga 1200
tggtcgaggt gaggtgtggc aggcttgaga tccaagatga agcgcgcaag accgtctgaa 1260
gataccttca accccgtgta tccatatgac acggaaaccg gtcctccaac tgtgcctttt 1320
cttactcctc cctttgtatc ccccaatggg tttcaagaga gtccccctgg ggtactctct 1380
ttgcgcctat ccgaacctct agttacctcc aatggcatgc ttgcgctcaa aatgggcaac 1440
ggcctctctc tggacgaggc cggcaacctt acctcccaaa atgtaaccac tgtgagccca 1500
cctctcaaaa aaaccaagtc aaacataaac ctggaaatat ctgcacccct cacagttacc 1560
tcagaagccc taactgtggc tgccgccgca cctctaatgg tcgcgggcaa cacactcacc 1620
atgcaatcac aggccccgct aaccgtgcac gactccaaac ttagcattgc cacccaagga 1680
cccctcacag tgtcagaagg aaagctagcc ctgcaaacat caggcccccct caccaccacc 1740
gatagcagta cccttactat cactgcctca ccccctctaa ctactgccac tggtagcttg 1800
ggcattgact tgaaagagcc catttataca caaaatggaa aactaggact aaagtacggg 1860
gctcctttgc atgtaacaga cgacctaaac actttgaccg tagcaactgg tccaggtgtg 1920
actattaata atacttcctt gcaaactaaa gttactggag ccttgggttt tgattcacaa 1980
ggcaatatgc aacttaatgt agcaggagga ctaaggattg attctcaaaa cagacgcctt 2040
atacttgatg ttagttatcc gtttgatgct caaaaccaac taaatctaag actaggacag 2100
ggccctcttt ttataaactc agcccacaac ttggatatta actacaacaa aggcctttac 2160
ttgtttacag cttcaaacaa ttccaaaaag cttgaggtta acctaagcac tgccaagggg 2220
ttgatgtttg acgctacagc catagccatt aatgcaggag atgggcttga atttggttca 2280
cctaatgcac caaacacaaa tcccctcaaa acaaaaattg gccatggcct agaatttgat 2340
tcaaacaagg ctatggttcc taaactagga actggcctta gttttgacag cacaggtgcc 2400
attacagtag gaaacaaaaa taatgataag ctaactttgt ggaccacacc agctccatct 2460
cctaactgta gactaaatgc agagaaagat gctaaactca ctttggtctt aacaaaatgt 2520
ggcagtcaaa tacttgctac agtttcagtt ttggctgtta aaggcagttt ggctccaata 2580
tctggaacag ttcaaagtgc tcatcttatt ataagatttg acgaaaatgg agtgctacta 2640
aacaattcct tcctggaccc agaatattgg aactttagaa atggagatct tactgaaggc 2700
acagcctata caaacgctgt tggatttatg cctaacctat cagcttatcc aaaatctcac 2760
ggtaaaactg ccaaaagtaa cattgtcagt caagtttact taaacggaga caaaactaaa 2820
cctgtaacac taaccattac actaaacggt acacaggaaa caggagacac aactccaagt 2880
gcatactcta tgtcattttc atgggactgg tctggccaca actacattaa tgaaatattt 2940
gccacatcct cttacacttt ttcatacatt gcccaagaat aaagaagcgg ccgctcgagc 3000
atgcatctag agggccctat tctatagtgt cacctaaatg ctagagctcg ctgatcagcc 3060
tcgactgtgc cttctagttg ccagccatct gttgtttgcc cctcccccgt gccttccttg 3120
accctggaag gtgccactcc cactgtcctt tcctaataaa atgaggaaat tgcatcgcat 3180
tgtctgagta ggtgtcattc tattctgggg ggtggggtgg ggcaggacag caagggggag 3240
gattgggaag acaatagcag gcatgctggg gatgcggtgg gctctatggc ttctgaggcg 3300
gaaagaacca gctggggctc tagggggtat ccccacgcgc cctgtagcgg cgcattaagc 3360
gcggcgggtg tggtggttac gcgcagcgtg accgctacac ttgccagcgc cctagcgccc 3420
gctcctttcg ctttcttccc ttcctttctc gccacgttcg ccggctttcc ccgtcaagct 3480

```
ctaaatcggg gcatcccttt agggttccga tttagtgctt tacggcacct cgaccccaaa 3540
aaacttgatt agggtgatgg ttcacgtagt gggccatcgc cctgatagac ggtttttcgc 3600
cctttgacgt tggagtccac gttctttaat agtggactct tgttccaaac tggaacaaca 3660
ctcaacccta tctcggtcta ttcttttgat ttataaggga ttttggggat ttcggcctat 3720
tggttaaaaa atgagctgat ttaacaaaaa tttaacgcga attaattctg tggaatgtgt 3780
gtcagttagg gtgtggaaag tccccaggct ccccaggcag gcagaagtat gcaaagcatg 3840
catctcaatt agtcagcaac caggtgtgga aagtccccag gctccccagc aggcagaagt 3900
atgcaaagca tgcatctcaa ttagtcagca accatagtcc cgcccctaac tccgcccatc 3960
ccgcccctaa ctccgcccag ttccgcccat tctccgcccc atggctgact aatttttttt 4020
atttatgcag aggccgaggc cgcctctgcc tctgagctat tccagaagta gtgaggaggc 4080
ttttttggag gcctaggctt ttgcaaaaag ctcccgggag cttgtatatc cattttcgga 4140
tctgatcaag agacaggatg aggatcgttt cgcatgattg aacaagatgg attgcacgca 4200
ggttctccgg ccgcttgggt ggagaggcta ttcggctatg actgggcaca acagacaatc 4260
ggctgctctg atgccgccgt gttccggctg tcagcgcagg ggcgcccggt tctttttgtc 4320
aagaccgacc tgtccggtgc cctgaatgaa ctgcaggacg aggcagcgcg gctatcgtgg 4380
ctggccacga cgggcgttcc ttgcgcagct gtgctcgacg ttgtcactga agcgggaagg 4440
gactggctgc tattgggcga agtgccgggg caggatctcc tgtcatctca ccttgctcct 4500
gccgagaaag tatccatcat ggctgatgca atgcggcggc tgcatacgct tgatccggct 4560
acctgcccat tcgaccacca agcgaaacat cgcatcgagc gagcacgtac tcggatggaa 4620
gccggtcttg tcgatcagga tgatctggac gaagagcatc aggggctcgc gccagccgaa 4680
ctgttcgcca ggctcaaggc gcgcatgccc gacggcgagg atctcgtcgt gacccatggc 4740
gatgcctgct tgccgaatat catggtggaa aatggccgct tttctggatt catcgactgt 4800
ggccggctgg gtgtggcgga ccgctatcag gacatagcgt tggctacccg tgatattgct 4860
gaagagcttg gcggcgaatg ggctgaccgc ttcctcgtgc tttacggtat cgccgctccc 4920
gattcgcagc gcatcgcctt ctatcgcctt cttgacgagt tcttctgagc gggactctgg 4980
ggttcgaaat gaccgaccaa gcgacgccca acctgccatc acgagatttc gattccaccg 5040
ccgccttcta tgaaaggttg ggcttcggaa tcgttttccg ggacgccggc tggatgatcc 5100
tccagcgcgg ggatctcatg ctggagttct tcgcccaccc caacttgttt attgcagctt 5160
ataatggtta caaataaagc aatagcatca caaatttcac aaataaagca tttttttcac 5220
tgcattctag ttgtggtttg tccaaactca tcaatgtatc ttatcatgtc tgtataccgt 5280
cgacctctag ctagagcttg gcgtaatcat ggtcatagct gtttcctgtg tgaaattgtt 5340
atccgctcac aattccacac aacatacgag ccggaagcat aaagtgtaaa gcctggggtg 5400
cctaatgagt gagctaactc acattaattg cgttgcgctc actgcccgct ttccagtcgg 5460
gaaacctgtc gtgccagctg cattaatgaa tcggccaacg cgcggggaga ggcggtttgc 5520
gtattgggcg ctcttccgct tcctcgctca ctgactcgct gcgctcggtc gttcggctgc 5580
ggcgagcggt atcagctcac tcaaaggcgg taatacggtt atccacagaa tcaggggata 5640
acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg 5700
cgttgctggc gtttttccat aggctccgcc cccctgacga gcatcacaaa aatcgacgct 5760
caagtcagag gtggcgaaac ccgacaggac tataaagata ccaggcgttt ccccctggaa 5820
gctccctcgt gcgctctcct gttccgaccc tgccgcttac cggatacctg tccgcctttc 5880
tcccttcggg aagcgtggcg ctttctcaat gctcacgctg taggtatctc agttcggtgt 5940
aggtcgttcg ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg 6000
ccttatccgg taactatcgt cttgagtcca acccggtaag acacgactta tcgccactgg 6060
cagcagccac tggtaacagg attagcagag cgaggtatgt aggcggtgct acagagttct 6120
tgaagtggtg gcctaactac ggctacacta gaaggacagt atttggtatc tgcgctctgc 6180
tgaagccagt taccttcgga aaaagagttg gtagctcttg atccggcaaa caaaccaccg 6240
ctggtagcgg tggttttttt gtttgcaagc agcagattac gcgcagaaaa aaaggatctc 6300
aagaagatcc tttgatcttt tctacggggt ctgacgctca gtggaacgaa aactcacgtt 6360
aagggatttt ggtcatgaga ttatcaaaaa ggatcttcac ctagatcctt ttaaattaaa 6420
aatgaagttt taaatcaatc taaagtatat atgagtaaac ttggtctgac agttaccaat 6480
gcttaatcag tgaggcacct atctcagcga tctgtctatt tcgttcatcc atagttgcct 6540
gactccccgt cgtgtagata actacgatac gggagggctt accatctggc cccagtgctg 6600
caatgatacc gcgagaccca cgctcaccgg ctccagattt atcagcaata aaccagccag 6660
ccggaagggc cgagcgcaga agtggtcctg caactttatc cgcctccatc cagtctatta 6720
attgttgccg ggaagctaga gtaagtagtt cgccagttaa tagtttgcgc aacgttgttg 6780
ccattgctac aggcatcgtg gtgtcacgct cgtcgtttgg tatggcttca ttcagctccg 6840
gttcccaacg atcaaggcga gttacatgat cccccatgtt gtgcaaaaaa gcggttagct 6900
ccttcggtcc tccgatcgtt gtcagaagta agttggccgc agtgttatca ctcatggtta 6960
tggcagcact gcataattct cttactgtca tgccatccgt aagatgcttt tctgtgactg 7020
gtgagtactc aaccaagtca ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc 7080
cggcgtcaat acgggataat accgcgccac atagcagaac tttaaaagtg ctcatcattg 7140
gaaaacgttc ttcggggcga aaactctcaa ggatcttacc gctgttgaga tccagttcga 7200
```

tgtaacccac tcgtgcaccc aactgatctt cagcatcttt tactttcacc agcgtttctg 7260
ggtgagcaaa aacaggaagg caaaatgccg caaaaaaggg aataagggcg acacggaaat 7320
gttgaatact catactcttc ctttttcaat attattgaag catttatcag ggttattgtc 7380
tcatgagcgg atacatattt gaatgtattt agaaaaataa acaaataggg gttccgcgca 7440
catttccccg aaaagtgcca cctgacgtc 7469

<210> 9
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 9
tgcttaagcg gccgcgaagg agaagtcc 28

<210> 10
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 10
ccgagctagc gactgaaaat gag 23

<210> 11
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 11
cctctcgaga gacagcaaga cac 23

<210> 12
<211> 11152
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid

<400> 12
aagcttgggc agaaatggtt gaactcccga gagtgtccta cacctagggg agaagcagcc 60
aaggggttgt ttcccaccaa ggacgacccg tctgcgcaca aacggatgag cccatcagac 120
aaagacatat tcattctctg ctgcaaactt ggcatagctc tgctttgcct ggggctattg 180
ggggaagttg cggttcgtgc tcgcagggct ctcacccttg actcttttaa tagctcttct 240
gtgcaagatt acaatctaaa caattcggag aactcgacct tcctcctgag gcaaggacca 300
cagccaactt cctcttacaa gccgcatcga ttttgtcctt cagaaataga aataagaatg 360
cttgctaaaa attatatttt taccaataag accaatccaa taggtagatt attagttact 420
atgttaagaa atgaatcatt atcttttagt actattttta ctcaaattca gaagttagaa 480
atgggaatag aaaatagaaa gagacgctca acctcaattg aagaacaggt gcaaggacta 540
ttgaccacag gcctagaagt aaaaaaggga aaaaagagtg tttttgtcaa aataggagac 600
aggtggtggc aaccagggac ttatagggga ccttacatct acagaccaac agatgccccc 660
ttaccatata caggaagata tgacttaaat tgggataggt gggttacagt caatggctat 720

aaagtgttat atagatccct cccttttcgt gaaagactcg ccagagctag acctccttgg 780
tgtatgttgt ctcaagaaga aaaagacgac atgaaacaac aggtacatga ttatatttat 840
ctaggaacag gaatgcactt ttggggaaag attttccata ccaaggaggg gacagtggct 900
ggactaatag aacattattc tgcaaaaact catggcatga gttattatga atagccttta 960
ttggcccaac cttgcggttc ccagggctta agtaagtttt tggttacaaa ctgttcttaa 1020
aacgaggatg tgagacaagt ggtttcctga cttggtttgg tatcaaaggt tctgatctga 1080
gctctgagtg ttctattttc ctatgttctt ttggaattta tccaaatctt atgtaaatgc 1140
ttatgtaaac caagatataa aagagtgctg atttttgag taaacttgca acagtcctaa 1200
cattcacctc ttgtgtgttt gtgtctgttc gccatcccgt ctccgctcgt cacttatcct 1260
tcactttcca gagggtcccc ccgcagaccc cggcgaccct caggtcggcc gactgcggca 1320
gctggcgccc gaacagggac cctcggataa gtgacccttg tctctatttc tactatttgg 1380
tgtttgtctt gtattgtctc tttcttgtct ggctatcatc acaagagcgg aacggactca 1440
ccatagggac caagctagcg actgaaaatg agacatatta tctgccacgg aggtgttatt 1500
accgaagaaa tggccgccag tcttttggac cagctgatcg aagaggtact ggctgataat 1560
cttccacctc ctagccattt tgaaccacct acccttcacg aactgtatga tttagacgtg 1620
acggcccccg aagatcccaa cgaggaggcg gtttcgcaga tttttcccga ctctgtaatg 1680
ttggcggtgc aggaagggat tgacttactc acttttccgc cggcgcccgg ttctccggag 1740
ccgcctcacc tttcccggca gcccgagcag ccggagcaga gagccttggg tccggtttct 1800
atgccaaacc ttgtaccgga ggtgatcgat cttacctgcc acgaggctgg ctttccaccc 1860
agtgacgacg aggatgaaga gggtgaggag tttgtgttag attatgtgga gcaccccggg 1920
cacggttgca ggtcttgtca ttatcaccgg aggaatacgg gggacccaga tattatgtgt 1980
tcgctttgct atatgaggac ctgtggcatg tttgtctaca gtaagtgaaa attatgggca 2040
gtgggtgata gagtggtggg tttggtgtgg taattttttt tttaattttt acagttttgt 2100
ggtttaaaga attttgtatt gtgatttttt taaaaggtcc tgtgtctgaa cctgagcctg 2160
agcccgagcc agaaccggag cctgcaagac ctacccgccg tcctaaaatg gcgcctgcta 2220
tcctgagacg cccgacatca cctgtgtcta gagaatgcaa tagtagtacg gatagctgtg 2280
actccggtcc ttctaacaca cctcctgaga tacacccggt ggtcccgctg tgccccatta 2340
aaccagttgc cgtgagagtt ggtgggcgtc gccaggctgt ggaatgtatc gaggacttgc 2400
ttaacgagcc tgggcaacct ttggacttga gctgtaaacg ccccaggcca taaggtgtaa 2460
acctgtgatt gcgtgtgtgg ttaacgcctt tgtttgctga atgagttgat gtaagtttaa 2520
taaagggtga gataatgttt aacttgcatg gcgtgttaaa tggggcgggg cttaaagggt 2580
atataatgcg ccgtgggcta atcttggtta catctgacct catggaggct tgggagtgtt 2640
tggaagattt ttctgctgtg cgtaacttgc tggaacagag ctctaacagt acctcttggt 2700
tttggaggtt tctgtggggc tcatcccagg caaagttagt ctgcagaatt aaggaggatt 2760
acaagtggga atttgaagag cttttgaaat cctgtggtga gctgtttgat tctttgaatc 2820
tgggtcacca ggcgcttttc caagagaagg tcatcaagac tttggatttt tccacaccgg 2880
ggcgcgctgc ggctgctgtt gctttttga gttttataaa ggataaatgg agcgaagaaa 2940
cccatctgag cgggggtac ctgctggatt ttctggccat gcatctgtgg agagcggttg 3000
tgagacacaa gaatcgcctg ctactgttgt cttccgtccg cccggcgata ataccgacgg 3060
aggagcagca gcagcagcag gaggaagcca ggcggcggcg gcaggagcag agcccatgga 3120
acccgagagc cggcctggac cctcgggaat gaatgttgta caggtggctg aactgtatcc 3180
agaactgaga cgcattttga caattacaga ggatgggcag gggctaaagg gggtaaagag 3240
ggagcggggg gcttgtgagg ctacagagga ggctaggaat ctagctttta gcttaatgac 3300
cagacaccgt cctgagtgta ttacttttca acagatcaag gataattgcg ctaatgagct 3360
tgatctgctg gcgcagaagt attccataga gcagctgacc acttactggc tgcagccagg 3420
ggatgatttt gaggaggcta ttagggtata tgcaaaggtg gcacttaggc cagattgcaa 3480
gtacaagatc agcaaacttg taaatatcag gaattgttgc tacatttctg ggaacggggc 3540
cgaggtggag atagatacgg aggatagggt ggcctttaga tgtagcatga taaatatgtg 3600
gccgggggtg cttggcatgg acggggtggt tattatgaat gtaaggttta ctggccccaa 3660
ttttagcggt acggttttcc tggccaatac caaccttatc ctacacggtg taagcttcta 3720
tgggtttaac aatacctgtg tggaagcctg gaccgatgta agggttcggg gctgtgcctt 3780
ttactgctgc tggaaggggg tggtgtgtcg ccccaaaagc agggcttcaa ttaagaaatg 3840
cctctttgaa aggtgtacct tgggtatcct gtctgagggt aactccaggg tgcgccacaa 3900
tgtggcctcc gactgtggtt gcttcatgct agtgaaaagc gtggctgtga ttaagcataa 3960
catggtatgt ggcaactgcg aggacagggc ctctcagatg ctgacctgct cggacggcaa 4020
ctgtcacctg ctgaagacca ttcacgtagc cagccactct cgcaaggcct ggccagtgtt 4080
tgagcataac atactgaccc gctgttcctt gcatttgggt aacaggaggg gggtgttcct 4140
accttaccaa tgcaatttga gtcacactaa gatattgctt gagcccgaga gcatgtccaa 4200
ggtgaacctg aacggggtgt ttgacatgac catgaagatc tggaaggtgc tgaggtacga 4260
tgagacccgc accaggtgca gaccctgcga gtgtggcggt aaacatatta ggaaccagcc 4320
tgtgatgctg gatgtgaccg aggagctgag gcccgatcac ttggtgctgg cctgcacccg 4380
cgctgagttt ggctctagcg atgaagatac agattgaggt actgaaatgt gtgggcgtgg 4440

cttaagggtg ggaaagaata tataaggtgg gggtcttatg tagttttgta tctgttttgc 4500
agcagccgcc gccgccatga gcaccaactc gtttgatgga agcattgtga gctcatattt 4560
gacaacgcgc atgcccccat gggccggggt gcgtcagaat gtgatgggct ccagcattga 4620
tggtcgcccc gtcctgcccg caaactctac taccttgacc tacgagaccg tgtctggaac 4680
gccgttggag actgcagcct ccgccgccgc ttcagccgct gcagccaccg cccgcgggat 4740
tgtgactgac tttgctttcc tgagcccgct tgcaagcagt gcagcttccc gttcatccgc 4800
ccgcgatgac aagttgacgg ctcttttggc acaattggat tctttgaccc gggaacttaa 4860
tgtcgtttct cagcagctgt tggatctgcg ccagcaggtt tctgccctga aggcttcctc 4920
ccctcccaat gcggtttaaa acataaataa aaaaccagac tctgtttgga tttggatcaa 4980
gcaagtgtct tgctgtctct cgagggatct ttgtgaagga accttacttc tgtggtgtga 5040
cataattgga caaactacct acagagattt aaagctctaa ggtaaatata aaatttttaa 5100
gtgtataatg tgttaaacta ctgattctaa ttgtttgtgt attttagatt ccaacctatg 5160
gaactgatga atgggagcag tggtggaatg cctttaatga ggaaaacctg ttttgctcag 5220
aagaaatgcc atctagtgat gatgaggcta ctgctgactc tcaacattct actcctccaa 5280
aaaagaagag aaaggtagaa gaccccaagg actttccttc agaattgcta agtttttga 5340
gtcatgctgt gtttagtaat agaactcttg cttgctttgc tatttacacc acaaaggaaa 5400
aagctgcact gctatacaag aaaattatgg aaaaatattc tgtaaccttt ataagtaggc 5460
ataacagtta taatcataac atactgtttt ttcttactcc acacaggcat agagtgtctg 5520
ctattaataa ctatgctcaa aaattgtgta cctttagctt tttaatttgt aaaggggtta 5580
ataaggaata tttgatgtat agtgccttga ctagagatca taatcagcca taccacattt 5640
gtagaggttt tacttgcttt aaaaaacctc ccacacctcc ccctgaacct gaaacataaa 5700
atgaatgcaa ttgttgttgt taacttgttt attgcagctt ataatggtta caaataaagc 5760
aatagcatca caaatttcac aaataaagca tttttttcac tgcattctag ttgtggtttg 5820
tccaaactca tcaatgtatc ttatcatgtc tggatccggc tgtggaatgt gtgtcagtta 5880
gggtgtggaa agtccccagg ctccccagca ggcagaagta tgcaaagcat gcatctcaat 5940
tagtcagcaa ccaggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc 6000
atgcatctca attagtcagc aaccatagtc ccgcccctaa ctccgcccat cccgccccta 6060
actccgccca gttccgccca ttctccgccc catggctgac taattttttt tatttatgca 6120
gaggccgagg ccgcctcggc ctctgagcta ttccagaagt agtgaggagg cttttttgga 6180
ggcctaggct tttgcaaaaa gcttggacac aagacaggct tgcgagatat gtttgagaat 6240
accactttat cccgcgtcag ggagaggcag tgcgtaaaaa gacgcggact catgtgaaat 6300
actggttttt agtgcgccag atctctataa tctcgcgcaa cctattttcc cctcgaacac 6360
tttttaagcc gtagataaac aggctgggac acttcacatg agcgaaaaat acatcgtcac 6420
ctgggacatg ttgcagatcc atgcacgtaa actcgcaagc cgactgatgc cttctgaaca 6480
atggaaaggc attattgccg taagccgtgg cggtctggta ccgggtgcgt tactggcgcg 6540
tgaactgggt attcgtcatg tcgataccgt ttgtatttcc agctacgatc acgacaacca 6600
gcgcgagctt aaagtgctga aacgcgcaga aggcgatggc gaaggcttca tcgttattga 6660
tgacctggtg gataccggtg gtactgcggt tgcgattcgt gaaatgtatc caaaagcgca 6720
ctttgtcacc atcttcgcaa aaccggctgg tcgtccgctg gttgatgact atgttgttga 6780
tatcccgcaa gatacctgga ttgaacagcc gtgggatatg ggcgtcgtat tcgtcccgcc 6840
aatctccggt cgctaatctt ttcaacgcct ggcactgccg ggcgttgttc tttttaactt 6900
caggcgggtt acaatagttt ccagtaagta ttctggaggc tgcatccatg acacaggcaa 6960
acctgagcga aaccctgttc aaaccccgct ttaaacatcc tgaaacctcg acgctagtcc 7020
gccgctttaa tcacggcgca caaccgcctg tgcagtcggc ccttgatggt aaaaccatcc 7080
ctcactggta tcgcatgatt aaccgtctga tgtggatctg gcgcggcatt gacccacgcg 7140
aaatcctcga cgtccaggca cgtattgtga tgagcgatgc cgaacgtacc gacgatgatt 7200
tatacgatac ggtgattggc taccgtggcg gcaactggat ttatgagtgg gccccggatc 7260
tttgtgaagg aaccttactt ctgtggtgtg acataattgg acaaactacc tacagagatt 7320
taaagctcta aggtaaatat aaaattttta agtgtataat gtgttaaact actgattcta 7380
attgtttgtg tattttagat tccaacctat ggaactgatg aatgggagca gtggtggaat 7440
gcctttaatg aggaaaacct gttttgctca gaagaaatgc catctagtga tgatgaggct 7500
actgctgact ctcaacattc tactcctcca aaaaagaaga gaaaggtaga agaccccaag 7560
gactttcctt cagaattgct aagtttttg agtcatgctg tgtttagtaa tagaactctt 7620
gcttgctttg ctatttacac cacaaaggaa aaagctgcac tgctatacaa gaaaattatg 7680
gaaaaatatt ctgtaacctt tataagtagg cataacagtt ataatcataa catactgttt 7740
tttcttactc cacacaggca tagagtgtct gctattaata actatgctca aaaattgtgt 7800
acctttagct ttttaatttg taaaggggtt aataaggaat atttgatgta tagtgccttg 7860
actagagatc ataatcagcc ataccacatt tgtagaggtt ttacttgctt taaaaaacct 7920
cccacacctc cccctgaacc tgaaacataa aatgaatgca attgttgttg ttaacttgtt 7980
tattgcagct tataatggtt acaaataaag caatagcatc acaaatttca caaataaagc 8040
attttttca ctgcattcta gttgtggttt gtccaaactc atcaatgtat cttatcatgt 8100
ctggatcccc aggaagctcc tctgtgtcct cataaaccct aacctcctct acttgagagg 8160

acattccaat cataggctgc ccatccaccc tctgtgtcct cctgttaatt aggtcactta 8220
acaaaaagga aattgggtag gggtttttca cagaccgctt tctaagggta atttaaaat 8280
atctgggaag tcccttccac tgctgtgttc cagaagtgtt ggtaaacagc ccacaaatgt 8340
caacagcaga aacatacaag ctgtcagctt tgcacaaggg cccaacaccc tgctcatcaa 8400
gaagcactgt ggttgctgtg ttagtaatgt gcaaaacagg aggcacattt tccccacctg 8460
tgtaggttcc aaaatatcta gtgttttcat ttttacttgg atcaggaacc cagcactcca 8520
ctggataagc attatcctta tccaaaacag ccttgtggtc agtgttcatc tgctgactgt 8580
caactgtagc atttttgggg gttacagttt gagcaggata tttggtcctg tagtttgcta 8640
acacaccctg cagctccaaa ggttccccac caacagcaaa aaaatgaaaa tttgaccctt 8700
gaatgggttt tccagcacca ttttcatgag tttttgtgt ccctgaatgc aagtttaaca 8760
tagcagttac cccaataacc tcagttttaa cagtaacagc ttccccacatc aaaatatttc 8820
cacaggttaa gtcctcattt aaattaggca aaggaattct tgaagacgaa agggcctcgt 8880
gatacgccta tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcaggtgg 8940
cacttttcgg ggaaatgtgc gcggaacccc tatttgttta tttttctaaa tacattcaaa 9000
tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa 9060
gagtatgagt attcaacatt tccgtgtcgc ccttattccc ttttttgcgg cattttgcct 9120
tcctgttttt gctcacccag aaacgctggt gaaagtaaaa gatgctgaag atcagttggg 9180
tgcacgagtg ggttacatcg aactggatct caacagcggt aagatccttg agagttttcg 9240
ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt 9300
atcccgtgtt gacgccgggc aagagcaact cggtcgccgc atacactatt ctcagaatga 9360
cttggttgag tactcaccag tcacagaaaa gcatcttacg gatggcatga cagtaagaga 9420
attatgcagt gctgccataa ccatgagtga taacactgcg gccaacttac ttctgacaac 9480
gatcggagga ccgaaggagc taaccgcttt tttgcacaac atgggggatc atgtaactcg 9540
ccttgatcgt tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac 9600
gatgcctgca gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct 9660
agcttcccgg caacaattaa tagactggat ggaggcggat aaagttgcag gaccacttct 9720
gcgctcggcc cttccggctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg 9780
gtctcgcggt atcattgcag cactggggcc agatggtaag ccctcccgta tcgtagttat 9840
ctacacgacg gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg 9900
tgcctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tactttagat 9960
tgatttaaaa cttcattttt aatttaaaag gatctaggtg aagatccttt ttgataatct 10020
catgaccaaa atcccttaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa 10080
gatcaaagga tcttcttgag atcctttttt tctgcgcgta atctgctgct tgcaaacaaa 10140
aaaaccaccg ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctcttttttcc 10200
gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta 10260
gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgctc tgctaatcct 10320
gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg 10380
atagttaccg gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca cacagcccag 10440
cttggagcga acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc 10500
cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg 10560
agagcgcacg agggagcttc cagggggaaa cgcctggtat ctttatagtc ctgtcgggtt 10620
tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg 10680
gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgctggc cttttgctca 10740
catgttcttt cctgcgttat cccctgattc tgtggataac cgtattaccg cctttgagtg 10800
agctgatacc gctcgccgca gccgaacgac cgagcgcagc gagtcagtga gcgaggaagc 10860
ggaagagcgc ctgatgcggt attttctcct tacgcatctg tgcggtattt cacaccgcat 10920
atggtgcact ctcagtacaa tctgctctga tgccgcatag ttaagccagt atacactccg 10980
ctatcgctac gtgactgggt catggctgcg ccccgacacc cgccaacacc cgctgacgcg 11040
ccctgacggg cttgtctgct cccggcatcc gcttacagac aagctgtgac cgtctccggg 11100
agctgcatgt gtcagaggtt ttcaccgtca tcaccgaaac gcgcgaggca gc 11152

<210> 13
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 13
gacggatcgg gagatctcc 19

<210> 14
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 14
ccgcctcaga agccatagag cc 22

<210> 15
<211> 14455
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid

<400> 15
aagcttgggc agaaatggtt gaactcccga gagtgtccta cacctagggg agaagcagcc 60
aaggggttgt ttcccaccaa ggacgacccg tctgcgcaca aacggatgag cccatcagac 120
aaagacatat tcattctctg ctgcaaactt ggcatagctc tgctttgcct ggggctattg 180
ggggaagttg cggttcgtgc tcgcagggct ctcacccttg actcttttaa tagctcttct 240
gtgcaagatt acaatctaaa caattcggag aactcgacct tcctcctgag gcaaggacca 300
cagccaactt cctcttacaa gccgcatcga ttttgtcctt cagaaataga aataagaatg 360
cttgctaaaa attatatttt taccaataag accaatccaa taggtagatt attagttact 420
atgttaagaa atgaatcatt atcttttagt actattttta ctcaaattca gaagttagaa 480
atgggaatag aaaatagaaa gagacgctca acctcaattg aagaacaggt gcaaggacta 540
ttgaccacag gcctagaagt aaaaaaggga aaaaagagtg tttttgtcaa aataggagac 600
aggtggtggc aaccagggac ttataggggа ccttacatct acagaccaac agatgccccc 660
ttaccatata caggaagata tgacttaaat tgggataggt gggttacagt caatggctat 720
aaagtgttat atagatccct cccttttcgt gaaagactcg ccagagctag acctccttgg 780
tgtatgttgt ctcaagaaga aaaagacgac atgaaacaac aggtacatga ttatatttat 840
ctaggaacag gaatgcactt ttggggaaag attttccata ccaaggaggg gacagtggct 900
ggactaatag aacattattc tgcaaaaact catggcatga gttattatga atagcccttta 960
ttggcccaac cttgcggttc ccagggctta agtaagtttt tggttacaaa ctgttcttaa 1020
aacgaggatg tgagacaagt ggtttcctga cttggtttgg tatcaaaggt tctgatctga 1080
gctctgagtg ttctattttc ctatgttctt ttggaattta tccaaatctt atgtaaatgc 1140
ttatgtaaac caagatataa aagagtgctg atttttgag taaacttgca acagtcctaa 1200
cattcacctc ttgtgtgttt gtgtctgttc gccatcccgt ctccgctcgt cacttatcct 1260
tcactttcca gagggtcccc ccgcagaccc cggcgaccct caggtcggcc gactgcggca 1320
gctggcgccc gaacagggac cctcggataa gtgacccttg tctctatttc tactatttgg 1380
tgtttgtctt gtattgtctc tttcttgtct ggctatcatc acaagagcgg aacggactca 1440
ccatagggac caagctagcg actgaaaatg agacatatta tctgccacgg aggtgttatt 1500
accgaagaaa tggccgccag tcttttggac cagctgatcg aagaggtact ggctgataat 1560
cttccacctc ctagccattt tgaaccacct acccttcacg aactgtatga tttagacgtg 1620
acggcccccg aagatcccaa cgaggaggcg gtttcgcaga tttttcccga ctctgtaatg 1680
ttggcggtgc aggaagggat tgacttactc acttttccgc cggcgcccgg ttctccggag 1740
ccgcctcacc tttcccggca gcccgagcag ccggagcaga gagccttggg tccggtttct 1800
atgccaaacc ttgtaccgga ggtgatcgat cttacctgcc acgaggctgg ctttccaccc 1860
agtgacgacg aggatgaaga gggtgaggag tttgtgttag attatgtgga gcacccgggg 1920
cacggttgca ggtcttgtca ttatcaccgg aggaatacgg gggacccaga tattatgtgt 1980
tcgctttgct atatgaggac ctgtggcatg tttgtctaca gtaagtgaaa attatgggca 2040
gtgggtgata gagtggtggg tttggtgtgg taattttttt tttaattttt acagttttgt 2100
ggtttaaaga attttgtatt gtgatttttt taaaaggtcc tgtgtctgaa cctgagcctg 2160
agcccgagcc agaaccggag cctgcaagac ctacccgccg tcctaaaatg gcgcctgcta 2220
tcctgagacg cccgacatca cctgtgtcta gagaatgcaa tagtagtacg gatagctgtg 2280
actccggtcc ttctaacaca cctcctgaga tacacccggt ggtcccgctg tgccccatta 2340
aaccagttgc cgtgagagtt ggtgggcgtc gccaggctgt ggaatgtatc gaggacttgc 2400
ttaacgagcc tgggcaacct ttggacttga gctgtaaacg ccccaggcca taaggtgtaa 2460

acctgtgatt gcgtgtgtgg ttaacgcctt tgtttgctga atgagttgat gtaagtttaa 2520
taaagggtga gataatgttt aacttgcatg gcgtgttaaa tggggcgggg cttaaagggt 2580
atataatgcg ccgtgggcta atcttggtta catctgacct catggaggct tgggagtgtt 2640
tggaagattt ttctgctgtg cgtaacttgc tggaacagag ctctaacagt acctcttggt 2700
tttggaggtt tctgtggggc tcatcccagg caaagttagt ctgcagaatt aaggaggatt 2760
acaagtggga atttgaagag cttttgaaat cctgtggtga gctgtttgat tctttgaatc 2820
tgggtcacca ggcgcttttc caagagaagg tcatcaagac tttggatttt tccacaccgg 2880
ggcgcgctgc ggctgctgtt gctttttga gttttataaa ggataaatgg agcgaagaaa 2940
cccatctgag cggggggtac ctgctggatt ttctggccat gcatctgtgg agagcggttg 3000
tgagacacaa gaatcgcctg ctactgttgt cttccgtccg cccggcgata ataccgacgg 3060
aggagcagca gcagcagcag gaggaagcca ggcggcggcg gcaggagcag agcccatgga 3120
acccgagagc cggcctggac cctcgggaat gaatgttgta caggtggctg aactgtatcc 3180
agaactgaga cgcattttga caattacaga ggatgggcag gggctaaagg gggtaaagag 3240
ggagcggggg gcttgtgagg ctacagagga ggctaggaat ctagctttta gcttaatgac 3300
cagacaccgt cctgagtgta ttacttttca acagatcaag gataattgcg ctaatgagct 3360
tgatctgctg gcgcagaagt attccataga gcagctgacc acttactggc tgcagccagg 3420
ggatgatttt gaggaggcta ttagggtata tgcaaaggtg gcacttaggc cagattgcaa 3480
gtacaagatc agcaaacttg taaatatcag gaattgttgc tacatttctg ggaacggggc 3540
cgaggtggag atagatacgg aggatagggt ggcctttaga tgtagcatga taaatatgtg 3600
gccgggggtg cttggcatgg acggggtggt tattatgaat gtaaggttta ctggccccaa 3660
tttagcggt acggttttcc tggccaatac caaccttatc ctacacggtg taagcttcta 3720
tgggtttaac aatacctgtg tggaagcctg gaccgatgta agggttcggg gctgtgcctt 3780
ttactgctgc tggaaggggg tggtgtgtcg ccccaaaagc agggcttcaa ttaagaaatg 3840
cctctttgaa aggtgtacct tgggtatcct gtctgagggt aactccaggg tgcgccacaa 3900
tgtggcctcc gactgtggtt gcttcatgct agtgaaaagc gtggctgtga ttaagcataa 3960
catggtatgt ggcaactgcg aggacagggc ctctcagatg ctgacctgct cggacggcaa 4020
ctgtcacctg ctgaagacca ttcacgtagc cagccactct cgcaaggcct ggccagtgtt 4080
tgagcataac atactgaccc gctgttcctt gcatttgggt aacaggaggg gggtgttcct 4140
accttaccaa tgcaatttga gtcacactaa gatattgctt gagcccgaga gcatgtccaa 4200
ggtgaacctg aacggggtgt ttgacatgac catgaagatc tggaaggtgc tgaggtacga 4260
tgagacccgc accaggtgca gaccctgcga gtgtggcggt aaacatatta ggaaccagcc 4320
tgtgatgctg gatgtgaccg aggagctgag gcccgatcac ttggtgctgg cctgcacccg 4380
cgctgagttt ggctctagcg atgaagatac agattgaggt actgaaatgt gtgggcgtgg 4440
cttaagggtg ggaaagaata tataaggtgg gggtcttatg tagttttgta tctgttttgc 4500
agcagccgcc gccgccatga gcaccaactc gtttgatgga agcattgtga gctcatattt 4560
gacaacgcgc atgcccccat gggccggggt gcgtcagaat gtgatgggct ccagcattga 4620
tggtcgcccc gtcctgcccg caaactctac taccttgacc tacgagaccg tgtctggaac 4680
gccgttggag actgcagcct ccgccgccgc ttcagccgct gcagccaccg cccgcgggat 4740
tgtgactgac tttgctttcc tgagcccgct tgcaagcagt gcagcttccc gttcatccgc 4800
ccgcgatgac aagttgacgg ctcttttggc acaattggat tctttgaccc gggaacttaa 4860
tgtcgtttct cagcagctgt tggatctgcg ccagcaggtt tctgccctga aggcttcctc 4920
ccctcccaat gcggtttaaa acataaataa aaaaccagac tctgtttgga tttggatcaa 4980
gcaagtgtct tgctgtctct cgagggatct ttgtgaagga accttacttc tgtggtgtga 5040
cataattgga caaactacct acagagattt aaagctctaa ggtaaatata aaatttttaa 5100
gtgtataatg tgttaaacta ctgattctaa ttgtttgtgt attttagatt ccaacctatg 5160
gaactgatga atgggagcag tggtggaatg cctttaatga ggaaaacctg ttttgctcag 5220
aagaaatgcc atctagtgat gatgaggcta ctgctgactc tcaacattct actcctccaa 5280
aaaagaagag aaaggtagaa gaccccaagg actttccttc agaattgcta agtttttga 5340
gtcatgctgt gtttagtaat agaactcttg cttgctttgc tatttacacc acaaaggaaa 5400
aagctgcact gctatacaag aaaattatgg aaaaatattc tgtaaccttt ataagtaggc 5460
ataacagtta taatcataac atactgtttt ttcttactcc acacaggcat agagtgtctg 5520
ctattaataa ctatgctcaa aaattgtgta cctttagctt tttaatttgt aaaggggtta 5580
ataaggaata tttgatgtat agtgccttga ctagagatca taatcagcca taccacattt 5640
gtagaggttt tacttgcttt aaaaaacctc ccacacctcc ccctgaacct gaaacataaa 5700
atgaatgcaa ttgttgttgt taacttgttt attgcagctt ataatggtta caaataaagc 5760
aatagcatca caaatttcac aaataaagca tttttttcac tgcattctag ttgtggtttg 5820
tccaaactca tcaatgtatc ttatcatgtc tggatccggc tgtggaatgt gtgtcagtta 5880
gggtgtggaa agtccccagg ctccccagca ggcagaagta tgcaaagcat gcatctcaat 5940
tagtcagcaa ccaggtgtgg aaagtcccca ggctccccag caggcagaag tatgcaaagc 6000
atgcatctca attagtcagc aaccatagtc ccgcccctaa ctccgcccat cccgccccta 6060
actccgccca gttccgccca ttctccgccc catggctgac taattttttt tatttatgca 6120
gaggccgagg ccgcctcggc ctctgagcta ttccagaagt agtgaggagg cttttttgga 6180

ggcctaggct tttgcaaaaa gcttggacac aagacaggct tgcgagatat gtttgagaat 6240
accactttat cccgcgtcag ggagaggcag tgcgtaaaaa gacgcggact catgtgaaat 6300
actggttttt agtgcgccag atctctataa tctcgcgcaa cctattttcc cctcgaacac 6360
tttttaagcc gtagataaac aggctgggac acttcacatg agcgaaaaat acatcgtcac 6420
ctgggacatg ttgcagatcc atgcacgtaa actcgcaagc cgactgatgc cttctgaaca 6480
atggaaaggc attattgccg taagccgtgg cggtctggta ccgggtgcgt tactggcgcg 6540
tgaactgggt attcgtcatg tcgataccgt ttgtatttcc agctacgatc acgacaacca 6600
gcgcgagctt aaagtgctga aacgcgcaga aggcgatggc gaaggcttca tcgttattga 6660
tgacctggtg gataccggtg gtactgcggt tgcgattcgt gaaatgtatc caaaagcgca 6720
ctttgtcacc atcttcgcaa aaccggctgg tcgtccgctg gttgatgact atgttgttga 6780
tatcccgcaa gatacctgga ttgaacagcc gtgggatatg ggcgtcgtat tcgtcccgcc 6840
aatctccggt cgctaatctt ttcaacgcct ggcactgccg ggcgttgttc tttttaactt 6900
caggcgggtt acaatagttt ccagtaagta ttctggaggc tgcatccatg acacaggcaa 6960
acctgagcga aaccctgttc aaaccccgct ttaaacatcc tgaaacctcg acgctagtcc 7020
gccgctttaa tcacggcgca caaccgcctg tgcagtcggc ccttgatggt aaaaccatcc 7080
ctcactggta tcgcatgatt aaccgtctga tgtggatctg gcgcggcatt gacccacgcg 7140
aaatcctcga cgtccaggca cgtattgtga tgagcgatgc cgaacgtacc gacgatgatt 7200
tatacgatac ggtgattggc taccgtggcg gcaactggat ttatgagtgg gccccggatc 7260
tttgtgaagg aaccttactt ctgtggtgtg acataattgg acaaactacc tacagagatt 7320
taaagctcta aggtaaatat aaaattttta agtgtataat gtgttaaact actgattcta 7380
attgtttgtg tattttagat tccaacctat ggaactgatg aatgggagca gtggtggaat 7440
gcctttaatg aggaaaacct gttttgctca gaagaaatgc catctagtga tgatgaggct 7500
actgctgact ctcaacattc tactcctcca aaaaagaaga gaaaggtaga agaccccaag 7560
gactttcctt cagaattgct aagtttttg agtcatgctg tgtttagtaa tagaactctt 7620
gcttgctttg ctatttacac cacaaaggaa aaagctgcac tgctatacaa gaaaattatg 7680
gaaaaatatt ctgtaacctt tataagtagg cataacagtt ataatcataa catactgttt 7740
tttcttactc cacacaggca tagagtgtct gctattaata actatgctca aaaattgtgt 7800
acctttagct ttttaatttg taaaggggtt aataaggaat atttgatgta tagtgccttg 7860
actagagatc ataatcagcc ataccacatt tgtagaggtt ttacttgctt taaaaaacct 7920
cccacacctc cccctgaacc tgaaacataa aatgaatgca attgttgttg ttaacttgtt 7980
tattgcagct tataatggtt acaaataaag caatagcatc acaaatttca caaataaagc 8040
atttttttca ctgcattcta gttgtggttt gtccaaactc atcaatgtat cttatcatgt 8100
ctggatcccc aggaagctcc tctgtgtcct cataaaccct aacctcctct acttgagagg 8160
acattccaat cataggctgc ccatccaccc tctgtgtcct cctgttaatt aggtcactta 8220
acaaaaagga aattgggtag ggtttttca cagaccgctt tctaagggta attttaaaat 8280
atctgggaag tcccttccac tgctgtgttc cagaagtgtt ggtaaacagc ccacaaatgt 8340
caacagcaga aacatacaag ctgtcagctt tgcacaaggg cccaacaccc tgctcatcaa 8400
gaagcactgt ggttgctgtg ttagtaatgt gcaaaacagg aggcacattt tccccacctg 8460
tgtaggttcc aaaatatcta gtgttttcat ttttacttgg atcaggaacc cagcactcca 8520
ctggataagc attatcctta tccaaaacag ccttgtggtc agtgttcatc tgctgactgt 8580
caactgtagc atttttggg gttacagttt gagcaggata tttggtcctg tagtttgcta 8640
acacaccctg cagctccaaa ggttccccac caacagcaaa aaaatgaaaa tttgaccctt 8700
gaatgggttt tccagcacca ttttcatgag ttttttgtgt ccctgaatgc aagtttaaca 8760
tagcagttac cccaataacc tcagttttaa cagtaacagc ttcccacatc aaaatatttc 8820
cacaggttaa gtcctcattt aaattaggca aaggaattct tgaagacgaa agggcctcgt 8880
gatacgccta tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcaggtgg 8940
cacttttcgg ggaaatgtgc gcggaacccc tatttgttta tttttctaaa tacattcaaa 9000
tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa 9060
gagtatgagt attcaacatt tccgtgtcgc ccttattccc ttttttgcgg cattttgcct 9120
tcctgttttt gctcacccag aaacgctggt gaaagtaaaa gatgctgaag atcagttggg 9180
tgcacgagtg ggttacatcg aactggatct caacagcggt aagatccttg agagttttcg 9240
ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt 9300
atcccgtgtt gacgccgggc aagagcaact cggtcgccgc atacactatt ctcagaatga 9360
cttggttgag tactcaccag tcacagaaaa gcatcttacg gatggcatga cagtaagaga 9420
attatgcagt gctgccataa ccatgagtga taacactgcg gccaacttac ttctgacaac 9480
gatcggagga ccgaaggagc taaccgcttt tttgcacaac atgggggatc atgtaactcg 9540
ccttgatcgt tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac 9600
gatgcctgca gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct 9660
agcttcccgg caacaattaa tagactggat ggaggcggat aaagttgcag gaccacttct 9720
gcgctcggcc cttccggctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg 9780
gtctcgcggt atcattgcag cactggggcc agatggtaag ccctcccgta tcgtagttat 9840
ctacacgacg gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg 9900

tgcctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tactttagat 9960
tgatttaaaa cttcattttt aatttaaaag gatctaggtg aagatccttt ttgataatct 10020
catgaccaaa atcccttaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa 10080
gatcaaagga tcttcttgag atcctttttt tctgcgcgta atctgctgct tgcaaacaaa 10140
aaaaccaccg ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctctttttcc 10200
gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta 10260
gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgctc tgctaatcct 10320
gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg 10380
atagttaccg gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca cacagcccag 10440
cttggagcga acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc 10500
cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg 10560
agagcgcacg agggagcttc cagggggaaa cgcctggtat ctttatagtc ctgtcgggtt 10620
tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg 10680
gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgctggc cttttgctca 10740
catgttcttt cctgcgttat cccctgattc tgtggataac cgtattaccg cctttgagtg 10800
agctgatacc gctcgccgca gccgaacgac cgagcgcagc gagtcagtga gcgaggaagc 10860
ggaagagcgc ctgatgcggt attttctcct tacgcatctg tgcggtattt cacaccgcat 10920
accgcctcag aagccataga gcccaccgca tccccagcat gcctgctatt gtcttcccaa 10980
tcctccccct tgctgtcctg ccccacccca cccccagaa tagaatgaca cctactcaga 11040
caatgcgatg caatttcctc attttattag gaaaggacag tgggagtggc accttccagg 11100
gtcaaggaag gcacggggga ggggcaaaca acagatggct ggcaactaga aggcacagtc 11160
gaggctgatc agcgagctct agcatttagg tgacactata gaatagggcc ctctagatgc 11220
atgctcgagc ggccgcttct ttattcttgg gcaatgtatg aaaaagtgta agaggatgtg 11280
gcaaatattt cattaatgta gttgtggcca gaccagtccc atgaaaatga catagagtat 11340
gcacttggag ttgtgtctcc tgtttcctgt gtaccgttta gtgtaatggt tagtgttaca 11400
ggtttagttt tgtctccgtt taagtaaact tgactgacaa tgttactttt ggcagtttta 11460
ccgtgagatt ttggataagc tgataggtta ggcataaatc caacagcgtt tgtataggct 11520
gtgccttcag taagatctcc atttctaaag ttccaatatt ctgggtccag gaaggaattg 11580
tttagtagca ctccattttc gtcaaatctt ataataagat gagcactttg aactgttcca 11640
gatattggag ccaaactgcc tttaacagcc aaaactgaaa ctgtagcaag tatttgactg 11700
ccacattttg ttaagaccaa agtgagttta gcatctttct ctgcatttag tctacagtta 11760
ggagatggag ctggtgtggt ccacaaagtt agcttatcat tatttttgtt tcctactgta 11820
atggcacctg tgctgtcaaa actaaggcca gttcctagtt taggaaccat agccttgttt 11880
gaatcaaatt ctaggccatg gccaattttt gttttgaggg gatttgtgtt tggtgcatta 11940
ggtgaaccaa attcaagccc atctcctgca ttaatggcta tggctgtagc gtcaaacatc 12000
aaccccttgg cagtgcttag gttaacctca agcttttgg aattgtttga agctgtaaac 12060
aagtaaaggc ctttgttgta gttaatatcc aagttgtggg ctgagtttat aaaaagaggg 12120
ccctgtccta gtcttagatt tagttggttt tgagcatcaa acggataact aacatcaagt 12180
ataaggcgtc tgttttgaga atcaatcctt agtcctcctg ctacattaag ttgcatattg 12240
ccttgtgaat caaaacccaa ggctccagta actttagttt gcaaggaagt attattaata 12300
gtcacacctg gaccagttgc tacggtcaaa gtgtttaggt cgtctgttac atgcaaagga 12360
gccccgtact ttagtcctag ttttccattt tgtgtataaa tgggctcttt caagtcaatg 12420
cccaagctac cagtggcagt agttagaggg ggtgaggcag tgatagtaag ggtactgcta 12480
tcggtggtgg tgagggggcc tgatgtttgc agggctagct ttccttctga cactgtgagg 12540
ggtccttggg tggcaatgct aagtttggag tcgtgcacgg ttagcggggc ctgtgattgc 12600
atggtgagtg tgttgcccgc gaccattaga ggtgcggcgg cagccacagt tagggcttct 12660
gaggtaactg tgaggggtgc agatatttcc aggtttatgt ttgacttggt ttttttgaga 12720
ggtgggctca cagtggttac attttgggag gtaaggttgc cggcctcgtc cagagagagg 12780
ccgttgccca ttttgagcgc aagcatgcca ttggaggtaa ctagaggttc ggataggcgc 12840
aaagagagta ccccaggggg actctcttga aacccattgg gggatacaaa gggaggagta 12900
agaaaaggca cagttggagg accggtttcc gtgtcatatg gatacacggg gttgaaggta 12960
tcttcagacg gtcttgcgcg cttcatcttg gatctcaagc ctgccacacc tcacctcgac 13020
catccgccgt ctcaagaccg cctactttaa ttacatcatc agcagcacct ccgccagaaa 13080
caaccccgac cgccacccgc tgccgcccgc cacggtgctc agcctacctt gcgactgtga 13140
ctggttagac gcctttctcg agaggttttc cgatccggtc gatgcggact cgctcaggtc 13200
cctcggtggc ggagtaccgt tcggaggccg acgggtttcc gatccaagag tactggaaag 13260
accgcgaaga gtttgtcctc aaccgcgagc ccaacagcga gctcgaattc agatccgagc 13320
tcggtaccaa gcttgggtct ccctatagtg agtcgtatta atttcgataa gccagtaagc 13380
agtgggttct ctagttagcc agagagctct gcttatatag acctcccacc gtacacgcct 13440
accgcccatt tgcgtcaatg gggcggagtt gttacgacat tttggaaagt cccgttgatt 13500
ttggtgccaa aacaaactcc cattgacgtc aatggggtgg agacttggaa atccccgtga 13560
gtcaaaccgc tatccacgcc cattgatgta ctgccaaaac cgcatcacca tggtaatagc 13620

gatgactaat acgtagatgt actgccaagt aggaaagtcc cataaggtca tgtactgggc 13680
ataatgccag gcgggccatt taccgtcatt gacgtcaata gggggcgtac ttggcatatg 13740
atacacttga tgtactgcca agtgggcagt ttaccgtaaa tagtccaccc attgacgtca 13800
atggaaagtc cctattggcg ttactatggg aacatacgtc attattgacg tcaatgggcg 13860
ggggtcgttg ggcggtcagc caggcgggcc atttaccgta agttatgtaa cgcggaactc 13920
catatatggg ctatgaacta atgaccccgt aattgattac tattaataac tagtcaataa 13980
tcaatgtcaa cgcgtatatc tggcccgtac atcgcgaagc agcgcaaaac gcctaaccct 14040
aagcagattc ttcatgcaat tgtcggtcaa gccttgcctt gttgtagctt aaattttgct 14100
cgcgcactac tcagcgacct ccaacacaca agcagggagc agatactggc ttaactatgc 14160
ggcatcagag cagattgtac tgagagtcga ccatagggga tcgggagatc tcccgatccg 14220
tctatggtgc actctcagta caatctgctc tgatgccgca tagttaagcc agtatacact 14280
ccgctatcgc tacgtgactg ggtcatggct gcgccccgac acccgccaac acccgctgac 14340
gcgccctgac gggcttgtct gctcccggca tccgcttaca gacaagctgt gaccgtctcc 14400
gggagctgca tgtgtcagag gttttcaccg tcatcaccga aacgcgcgag gcagc 14455

<210> 16
<211> 10610
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid

<400> 16
gacggatcgg gagatccgcg cggtacacag aattcaggag acacaactcc aagtgcatac 60
tctatgtcat tttcatggga ctggtctggc cacaactaca ttaatgaaat atttgccaca 120
tcctcttaca ctttttcata cattgcccaa gaataaagaa tcgtttgtgt tatgtttcaa 180
cgtgtttatt tttcaattgc agaaaatttc aagtcatttt tcattcagta gtatagcccc 240
accaccacat agcttataca gatcaccgta ccttaatcaa actcacagaa ccctagtatt 300
caacctgcca cctccctccc aacacacaga gtacacagtc ctttctcccc ggctggcctt 360
aaaaagcatc atatcatggg taacagacat attcttaggt gttatattcc acacggtttc 420
ctgtcgagcc aaacgctcat cagtgatatt aataaactcc ccgggcagct cacttaagtt 480
catgtcgctg tccagctgct gagccacagg ctgctgtcca acttgcggtt gcttaacggg 540
cggcgaagga gaagtccacg cctacatggg ggtagagtca taatcgtgca tcaggatagg 600
gcggtggtgc tgcagcagcg cgcgaataaa ctgctgccgc cgccgctccg tcctgcagga 660
atacaacatg gcagtggtct cctcagcgat gattcgcacc gcccgcagca taaggcgcct 720
tgtcctccgg gcacagcagc gcaccctgat ctcacttaaa tcagcacagt aactgcagca 780
cagcaccaca atattgttca aaatcccaca gtgcaaggcg ctgtatccaa agctcatggc 840
ggggaccaca gaacccacgt ggccatcata ccacaagcgc aggtagatta agtggcgacc 900
cctcataaac acgctggaca taaacattac ctcttttggc atgttgtaat tcaccacctc 960
ccggtaccat ataaacctct gattaaacat ggcgccatcc accaccatcc taaaccagct 1020
ggccaaaacc tgcccgccgg ctatacactg cagggaaccg ggactggaac aatgacagtg 1080
gagagcccag gactcgtaac catggatcat catgctcgtc atgatatcaa tgttggcaca 1140
acacaggcac acgtgcatac acttcctcag gattacaagc tcctcccgcg ttagaaccat 1200
atcccaggga acaaccatt cctgaatcag cgtaaatccc acactgcagg gaagacctcg 1260
cacgtaactc acgttgtgca ttgtcaaagt gttacattcg ggcagcagcg gatgatcctc 1320
cagtatggta gcgcgggttt ctgtctcaaa aggaggtaga cgatccctac tgtacggagt 1380
gcgccgagac aaccgagatc gtgttggtcg tagtgtcatg ccaaatggaa cgccggacgt 1440
agtcatattt cctgaagcaa aaccaggtgc gggcgtgaca aacagatctg cgtctccggt 1500
ctcgccgctt agatcgctct gtgtagtagt tgtagtatat ccactctctc aaagcatcca 1560
ggcgcccct ggcttcgggt tctatgtaaa ctccttcatg cgccgctgcc ctgataacat 1620
ccaccaccgc agaataagcc acacccagcc aacctacaca ttcgttctgc gagtcacaca 1680
cgggaggagc gggaagagct ggaagaacca tgtttttttt tttattccaa aagattatcc 1740
aaaacctcaa aatgaagatc tattaagtga acgcgctccc ctccggtggc gtggtcaaac 1800
tctacagcca aagaacagat aatggcattt gtaagatgtt gcacaatggc ttccaaaagg 1860
caaacggccc tcacgtccaa gtggacgtaa aggctaaacc cttcagggtg aatctcctct 1920
ataaacattc cagcaccttc aaccatgccc aaataattct catctcgcca ccttctcaat 1980
atatctctaa gcaaatcccg aatattaagt ccggccattg taaaaatctg ctccagagcg 2040
ccctccacct tcagcctcaa gcagcgaatc atgattgcaa aaattcaggt tcctcacaga 2100
cctgtataag attcaaaagc ggaacattaa caaaaatacc gcgatcccgt aggtcccttc 2160
gcagggccag ctgaacataa tcgtgcaggt ctgcacggac cagcgcggcc acttccccgc 2220

caggaacctt gacaaaagaa cccacactga ttatgacacg catactcgga gctatgctaa 2280
ccagcgtagc cccgatgtaa gctttgttgc atgggcggcg atataaaatg caaggtgctg 2340
ctcaaaaaat caggcaaagc ctcgcgcaaa aaagaaagca catcgtagtc atgctcatgc 2400
agataaaggc aggtaagctc cggaaccacc acagaaaaag acaccatttt tctctcaaac 2460
atgtctgcgg gtttctgcat aaacacaaaa taaaataaca aaaaaacatt taaacattag 2520
aagcctgtct tacaacagga aaaacaaccc ttataagcat aagacggact acggccatgc 2580
cggcgtgacc gtaaaaaaac tggtcaccgt gattaaaaag caccaccgac agctcctcgg 2640
tcatgtccgg agtcataatg taagactcgg taaacacatc aggttgattc atcggtcagt 2700
gctaaaaagc gaccgaaata gcccggggga atacataccc gcaggcgtag agacaacatt 2760
acagccccca taggaggtat aacaaaatta ataggagaga aaaacacata aacacctgaa 2820
aaaccctcct gcctaggcaa aatagcaccc tcccgctcca gaacaacata cagcgcttca 2880
cagcggcagc ctaacagtca gccttaccag taaaaaagaa aacctattaa aaaaacacca 2940
ctcgacacgg caccagctca atcagtcaca gtgtaaaaaa gggccaagtg cagagcgagt 3000
atatatagga ctaaaaaatg acgtaacggt taaagtccac aaaaaacacc cagaaaaccg 3060
cacgcgaacc tacgcccaga aacgaaagcc aaaaaaccca caacttcctc aaatcgtcac 3120
ttccgttttc ccacgttacg taacttcccg gatcctctcc cgatccccta tggtcgactc 3180
tcagtacaat ctgctctgat gccgcatagt taagccagta tctgctccct gcttgtgtgt 3240
tggaggtcgc tgagtagtgc gcgagcaaaa tttaagctac aacaaggcaa ggcttgaccg 3300
acaattgcat gaagaatctg cttagggtta ggcgttttgc gctgcttcgc gatgtacggg 3360
ccagatatac gcgttgacat tgattattga ctagttatta atagtaatca attacggggt 3420
cattagttca tagcccatat atggagttcc gcgttacata acttacggta aatggcccgc 3480
ctggctgacc gcccaacgac ccccgcccat tgacgtcaat aatgacgtat gttcccatag 3540
taacgccaat agggactttc cattgacgtc aatgggtgga ctatttacgg taaactgccc 3600
acttggcagt acatcaagtg tatcatatgc caagtacgcc ccctattgac gtcaatgacg 3660
gtaaatggcc cgcctggcat tatgcccagt acatgacctt atgggacttt cctacttggc 3720
agtacatcta cgtattagtc atcgctatta ccatggtgat gcggttttgg cagtacatca 3780
atgggcgtgg atagcggttt gactcacggg gatttccaag tctccacccc attgacgtca 3840
atgggagttt gttttggcac caaaatcaac gggactttcc aaaatgtcgt aacaactccg 3900
ccccattgac gcaaatgggc ggtaggcgtg tacggtggga ggtctatata agcagagctc 3960
tctggctaac tagagaaccc actgcttact ggcttatcga aattaatacg actcactata 4020
gggagaccca agcttggtac cgagctcgga tctgaattcg agctcgctgt tgggctcgcg 4080
gttgaggaca aactcttcgc ggtctttcca gtactcttgg atcggaaacc cgtcggcctc 4140
cgaacggtac tccgccaccg agggacctga gcgagtccgc atcgaccgga tcggaaaacc 4200
tctcgagaaa ggcgtctaac cagtcacagt cgcaaggtag gctgagcacc gtggcgggcg 4260
gcagcgggtg gcggtcgggg ttgtttctgg cggaggtgct gctgatgatg taattaaagt 4320
aggcggtctt gagacggcgg atggtcgagg tgaggtgtgg caggcttgag atccaagatg 4380
aagcgcgcaa gaccgtctga agataccttc aaccccgtgt atccatatga cacggaaacc 4440
ggtcctccaa ctgtgccttt tcttactcct ccctttgtat cccccaatgg gtttcaagag 4500
agtccccctg gggtactctc tttgcgccta tccgaacctc tagttacctc caatggcatg 4560
cttgcgctca aaatgggcaa cggcctctct ctggacgagg ccggcaacct tacctcccaa 4620
aatgtaacca ctgtgagccc acctctcaaa aaaaccaagt caaacataaa cctggaaata 4680
tctgcacccc tcacagttac ctcagaagcc ctaactgtgg ctgccgccgc acctctaatg 4740
gtcgcgggca acacactcac catgcaatca caggccccgc taaccgtgca cgactccaaa 4800
cttagcattg ccacccaagg acccctcaca gtgtcagaag gaaagctagc cctgcaaaca 4860
tcaggccccc tcaccaccac cgatagcagt acccttacta tcactgcctc acccccteta 4920
actactgcca ctggtagctt gggcattgac ttgaaagagc ccatttatac acaaaatgga 4980
aaactaggac taaagtacgg ggctcctttg catgtaacag acgacctaaa cactttgacc 5040
gtagcaactg gtccaggtgt gactattaat aatacttcct tgcaaactaa agttactgga 5100
gccttgggtt ttgattcaca aggcaatatg caacttaatg tagcaggagg actaaggatt 5160
gattctcaaa acagacgcct tatacttgat gttagttatc cgtttgatgc tcaaaaccaa 5220
ctaaatctaa gactaggaca gggccctctt tttataaact cagcccacaa cttggatatt 5280
aactacaaca aaggccttta cttgtttaca gcttcaaaca attccaaaaa gcttgaggtt 5340
aacctaagca ctgccaaggg gttgatgttt gacgctacag ccatagccat taatgcagga 5400
gatgggcttg aatttggttc acctaatgca ccaaacacaa atcccctcaa aacaaaaatt 5460
ggccatggcc tagaatttga ttcaaacaag gctatggttc ctaaactagg aactggcctt 5520
agttttgaca gcacaggtgc cattacagta ggaaacaaaa ataatgataa gctaactttg 5580
tggaccacac cagctccatc tcctaactgt agactaaatg cagagaaaga tgctaaactc 5640
actttggtct taacaaaatg tggcagtcaa atacttgcta cagtttcagt tttggctgtt 5700
aaaggcagtt tggctccaat atctggaaca gttcaaagtg ctcatcttat tataagattt 5760
gacgaaaatg gagtgctact aaacaattcc ttcctggacc cagaatattg gaactttaga 5820
aatggagatc ttactgaagg cacagcctat acaaacgctg ttggatttat gcctaaccta 5880
tcagcttatc caaaatctca cggtaaaact gccaaaagta acattgtcag tcaagtttac 5940

ttaaacggag acaaaactaa acctgtaaca ctaaccatta cactaaacgg tacacaggaa 6000
acaggagaca caactccaag tgcatactct atgtcatttt catgggactg gtctggccac 6060
aactacatta atgaaatatt tgccacatcc tcttacactt tttcatacat tgcccaagaa 6120
taaagaagcg gccgctcgag catgcatcta gagggcccta ttctatagtg tcacctaaat 6180
gctagagctc gctgatcagc ctcgactgtg ccttctagtt gccagccatc tgttgtttgc 6240
ccctcccccg tgccttcctt gaccctggaa ggtgccactc ccactgtcct ttcctaataa 6300
aatgaggaaa ttgcatcgca ttgtctgagt aggtgtcatt ctattctggg gggtggggtg 6360
gggcaggaca gcaaggggga ggattgggaa gacaatagca ggcatgctgg ggatgcggtg 6420
ggctctatgg cttctgaggc ggaaagaacc agctggggct ctagggggta tccccacgcg 6480
ccctgtagcg gcgcattaag cgcggcgggt gtggtggtta cgcgcagcgt gaccgctaca 6540
cttgccagcg ccctagcgcc cgctcctttc gctttcttcc cttcctttct cgccacgttc 6600
gccggctttc cccgtcaagc tctaaatcgg ggcatccctt tagggttccg atttagtgct 6660
ttacggcacc tcgaccccaa aaaacttgat tagggtgatg gttcacgtag tgggccatcg 6720
ccctgataga cggtttttcg ccctttgacg ttggagtcca cgttctttaa tagtggactc 6780
ttgttccaaa ctggaacaac actcaaccct atctcggtct attcttttga tttataaggg 6840
attttgggga tttcggccta ttggttaaaa aatgagctga tttaacaaaa atttaacgcg 6900
aattaattct gtggaatgtg tgtcagttag ggtgtggaaa gtccccaggc tccccaggca 6960
ggcagaagta tgcaaagcat gcatctcaat tagtcagcaa ccaggtgtgg aaagtcccca 7020
ggctccccag caggcagaag tatgcaaagc atgcatctca attagtcagc aaccatagtc 7080
ccgcccctaa ctccgcccat cccgccccta actccgccca gttccgccca ttctccgccc 7140
catggctgac taattttttt tatttatgca gaggccgagg ccgcctctgc ctctgagcta 7200
ttccagaagt agtgaggagg cttttttgga ggcctaggct tttgcaaaaa gctcccggga 7260
gcttgtatat ccattttcgg atctgatcaa gagacaggat gaggatcgtt tcgcatgatt 7320
gaacaagatg gattgcacgc aggttctccg gccgcttggg tggagaggct attcggctat 7380
gactgggcac aacagacaat cggctgctct gatgccgccg tgttccggct gtcagcgcag 7440
gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga actgcaggac 7500
gaggcagcgc ggctatcgtg gctggccacg acgggcgttc cttgcgcagc tgtgctcgac 7560
gttgtcactg aagcgggaag ggactggctg ctattgggcg aagtgccggg gcaggatctc 7620
ctgtcatctc accttgctcc tgccgagaaa gtatccatca tggctgatgc aatgcggcgg 7680
ctgcatacgc ttgatccggc tacctgccca ttcgaccacc aagcgaaaca tcgcatcgag 7740
cgagcacgta ctcggatgga agccggtctt gtcgatcagg atgatctgga cgaagagcat 7800
caggggctcg cgccagccga actgttcgcc aggctcaagg cgcgcatgcc cgacggcgag 7860
gatctcgtcg tgacccatgg cgatgcctgc ttgccgaata tcatggtgga aaatggccgc 7920
ttttctggat tcatcgactg tggccggctg ggtgtggcgg accgctatca ggacatagcg 7980
ttggctaccc gtgatattgc tgaagagctt ggcggcgaat gggctgaccg cttcctcgtg 8040
ctttacggta tcgccgctcc cgattcgcag cgcatcgcct tctatcgcct tcttgacgag 8100
ttcttctgag cgggactctg gggttcgaaa tgaccgacca agcgacgccc aacctgccat 8160
cacgagattt cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc 8220
gggacgccgg ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc 8280
ccaacttgtt tattgcagct tataatggtt acaaataaag caatagcatc acaaatttca 8340
caaataaagc atttttttca ctgcattcta gttgtggttt gtccaaactc atcaatgtat 8400
cttatcatgt ctgtataccg tcgacctcta gctagagctt ggcgtaatca tggtcatagc 8460
tgtttcctgt gtgaaattgt tatccgctca caattccaca caacatacga gccggaagca 8520
taaagtgtaa agcctggggt gcctaatgag tgagctaact cacattaatt gcgttgcgct 8580
cactgcccgc tttccagtcg ggaaacctgt cgtgccagct gcattaatga atcggccaac 8640
gcgcggggag aggcggtttg cgtattgggc gctcttccgc ttcctcgctc actgactcgc 8700
tgcgctcggt cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt 8760
tatccacaga atcaggggat aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg 8820
ccaggaaccg taaaaaggcc gcgttgctgg cgtttttcca taggctccgc ccccctgacg 8880
agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa cccgacagga ctataaagat 8940
accaggcgtt tccccctgga agctccctcg tgcgctctcc tgttccgacc ctgccgctta 9000
ccggatacct gtccgccttt ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct 9060
gtaggtatct cagttcggtg taggtcgttc gctccaagct gggctgtgtg cacgaacccc 9120
ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc aacccggtaa 9180
gacacgactt atcgccactg gcagcagcca ctggtaacag gattagcaga gcgaggtatg 9240
taggcggtgc tacagagttc ttgaagtggt ggcctaacta cggctacact agaaggacag 9300
tatttggtat ctgcgctctg ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt 9360
gatccggcaa acaaaccacc gctggtagcg gtggtttttt tgtttgcaag cagcagatta 9420
cgcgcagaaa aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc 9480
agtggaacga aaactcacgt taagggattt tggtcatgag attatcaaaa aggatcttca 9540
cctagatcct tttaaattaa aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa 9600
cttggtctga cagttaccaa tgcttaatca gtgaggcacc tatctcagcg atctgtctat 9660

ttcgttcatc catagttgcc tgactccccg tcgtgtagat aactacgata cgggagggct 9720
taccatctgg ccccagtgct gcaatgatac cgcgagaccc acgctcaccg gctccagatt 9780
tatcagcaat aaaccagcca gccggaaggg ccgagcgcag aagtggtcct gcaactttat 9840
ccgcctccat ccagtctatt aattgttgcc gggaagctag agtaagtagt tcgccagtta 9900
atagtttgcg caacgttgtt gccattgcta caggcatcgt ggtgtcacgc tcgtcgtttg 9960
gtatggcttc attcagctcc ggttcccaac gatcaaggcg agttacatga tcccccatgt 10020
tgtgcaaaaa agcggttagc tccttcggtc ctccgatcgt tgtcagaagt aagttggccg 10080
cagtgttatc actcatggtt atggcagcac tgcataattc tcttactgtc atgccatccg 10140
taagatgctt ttctgtgact ggtgagtact caaccaagtc attctgagaa tagtgtatgc 10200
ggcgaccgag ttgctcttgc ccggcgtcaa tacgggataa taccgcgcca catagcagaa 10260
ctttaaaagt gctcatcatt ggaaaacgtt cttcggggcg aaaactctca aggatcttac 10320
cgctgttgag atccagttcg atgtaaccca ctcgtgcacc caactgatct tcagcatctt 10380
ttactttcac cagcgtttct gggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg 10440
gaataagggc gacacggaaa tgttgaatac tcatactctt cctttttcaa tattattgaa 10500
gcatttatca gggttattgt ctcatgagcg gatacatatt tgaatgtatt tagaaaaata 10560
aacaaatagg ggttccgcgc acatttcccc gaaaagtgcc acctgacgtc 10610

<210> 17
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 17
tgtacaccgg atccggcgca cacc 24

<210> 18
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 18
cacaacgagc tcaattaatt aattgccaca tcctc 35

<210> 19
<211> 4
<212> PRT
<213> adenovirus

<400> 19
Thr Leu Trp Thr
1

<210> 20
<211> 12
<212> PRT
<213> adenovirus

<400> 20
Pro Ser Ala Ser Ala Ser Ala Ser Ala Pro Gly Ser
1 5 10

<210> 22
<211> 327
<212> DNA

<213> adenovirus

<400> 22
agatctgaat tcgagctcgc tgttgggctc gcggttgagg acaaactctt cgcggtcttt 60
ccagtactct tggatcggaa acccgtcggc ctccgaacgg tactccgcca ccgagggacc 120
tgagcgagtc cgcatcgacc ggatcggaaa acctctcgag aaaggcgtct aaccagtcac 180
agtcgcaagg taggctgagc accgtggcgg gcggcagcgg gtggcggtcg ggttgtttc 240
tggcggaggt gctgctgatg atgtaattaa agtaggcggt cttgagacgg cggatggtcg 300
aggtgaggtg tggcaggctt gagatct 327

<210> 23
<211> 32480
<212> DNA
<213> adenovirus

<400> 23
catcatcaat aatatacctt attttggatt gaagccaata tgataatgag ggggtggagt 60
ttgtgacgtg gcgcggggcg tgggaacggg gcgggtgacg tagtagtgtg gcggaagtgt 120
gatgttgcaa gtgtggcgga acacatgtaa gcgacggatg tggcaaaagt gacgtttttg 180
gtgtgcgccg gtgtacacag gaagtgacaa ttttcgcgcg gttttaggcg gatgttgtag 240
taaatttggg cgtaaccgag taagatttgg ccattttcgc gggaaaactg aataagagga 300
agtgaaatct gaataatttt gtgttactca tagcgcgtaa tctctagcat cgatgtcgac 360
aagcttgaat tcgattaatg tgagttagct cactcattag gcaccccagg ctttacactt 420
tatgcttccg gctcgtatgt tgtgtggaat tgtgagcgga taacaatttc acacaggaaa 480
cagctatgac catgattacg aattcggcgc agcaccatgg cctgaaataa cctctgaaag 540
aggaacttgg ttaggtacct tctgaggcgg aaagaaccag ctgtggaatg tgtgtcagtt 600
agggtgtgga aagtccccag gctccccagc aggcagaagt atgcaaagca tgcatctcaa 660
ttagtcagca accaggtgtg gaaagtcccc aggctcccca gcaggcagaa gtatgcaaag 720
catgcatctc aattagtcag caaccatagt cccgccccta actccgccca tcccgcccct 780
aactccgccc agttccgccc attctccgcc ccatggctga ctaatttttt ttatttatgc 840
agaggccgag gccgcctcgg cctctgagct attccagaag tagtgaggag gcttttttgg 900
aggcctaggc ttttgcaaaa agcttgggat ctctataatc tcgcgcaacc tattttcccc 960
tcgaacactt tttaagccgt agataaacag gctgggacac ttcacatgag cgaaaaatac 1020
atcgtcacct gggacatgtt gcagatccat gcacgtaaac tcgcaagccg actgatgcct 1080
tctgaacaat ggaaaggcat tattgccgta agccgtggcg gtctggtacc ggtgggtgaa 1140
gaccagaaac agcacctcga actgagccgc gatattgccc agcgtttcaa cgcgctgtat 1200
ggcgagatcg atcccgtcgt tttacaacgt cgtgactggg aaaaccctgg cgttacccaa 1260
cttaatcgcc ttgcagcaca tccccctttc gccagctggc gtaatagcga agaggcccgc 1320
accgatcgcc cttcccaaca gttgcgcagc ctgaatggcg aatggcgctt tgcctggttt 1380
ccggcaccag aagcggtgcc ggaaagctgg ctggagtgcg atcttcctga ggccgatact 1440
gtcgtcgtcc cctcaaactg gcagatgcac ggttacgatg cgcccatcta caccaacgta 1500
acctatccca ttacggtcaa tccgccgttt gttcccacgg agaatccgac gggttgttac 1560
tcgctcacat ttaatgttga tgaaagctgg ctacaggaag gccagacgcg aattattttt 1620
gatggcgtta actcggcgtt tcatctgtgg tgcaacgggc gctgggtcgg ttacggccag 1680
gacagtcgtt tgccgtctga atttgacctg agcgcatttt tacgcgccgg agaaaaccgc 1740
ctcgcggtga tggtgctgcg ttggagtgac ggcagttatc tggaagatca ggatatgtgg 1800
cggatgagcg gcattttccg tgacgtctcg ttgctgcata aaccgactac acaaatcagc 1860
gatttccatg ttgccactcg ctttaatgat gatttcagcc gcgctgtact ggaggctgaa 1920
gttcagatgt gcggcgagtt gcgtgactac ctacgggtaa cagtttcttt atggcagggt 1980
gaaacgcagg tcgccagcgg caccgcgcct ttcggcggtg aaattatcga tgagcgtggt 2040
ggttatgccg atcgcgtcac actacgtctg aacgtcgaaa acccgaaact gtggagcgcc 2100
gaaatcccga atctctatcg tgcggtggtt gaactgcaca ccgccgacgg cacgctgatt 2160
gaagcagaag cctgcgatgt cggtttccgc gaggtgcgga ttgaaaatgg tctgctgctg 2220
ctgaacggca agccgttgct gattcgaggc gttaaccgtc acgagcatca tcctctgcat 2280
ggtcaggtca tggatgagca gacgatggtg caggatatcc tgctgatgaa gcagaacaac 2340
tttaacgccg tgcgctgttc gcattatccg aaccatccgc tgtggtacac gctgtgcgac 2400
cgctacggcc tgtatgtggt ggatgaagcc aatattgaaa cccacggcat ggtgccaatg 2460
aatcgtctga ccgatgatcc gcgctggcta ccggcgatga gcgaacgcgt aacgcgaatg 2520
gtgcagcgcg atcgtaatca cccgagtgtg atcatctggt cgctggggaa tgaatcaggc 2580
cacggcgcta atcacgacgc gctgtatcgc tggatcaaat ctgtcgatcc ttcccgcccg 2640
gtgcagtatg aaggcggcgg agccgacacc acggccaccg atattatttg cccgatgtac 2700
gcgcgcgtgg atgaagacca gcccttcccg gctgtgccga aatggtccat caaaaaatgg 2760

```
ctttcgctac ctggagagac gcgcccgctg atcctttgcg aatacgccca cgcgatgggt 2820
aacagtcttg gcggtttcgc taaatactgg caggcgtttc gtcagtatcc ccgtttacag 2880
ggcggcttcg tctgggactg ggtggatcag tcgctgatta aatatgatga aaacggcaac 2940
ccgtggtcgg cttacggcgg tgattttggc gatacgccga acgatcgcca gttctgtatg 3000
aacggtctgg tctttgccga ccgcacgccg catccagcgc tgacggaagc aaaacaccag 3060
cagcagtttt tccagttccg tttatccggg caaaccatcg aagtgaccag cgaatacctg 3120
ttccgtcata gcgataacga gctcctgcac tggatggtgg cgctggatgg taagccgctg 3180
gcaagcggtg aagtgcctct ggatgtcgct ccacaaggta aacagttgat tgaactgcct 3240
gaactaccgc agccggagag cgccgggcaa ctctggctca cagtacgcgt agtgcaaccg 3300
aacgcgaccg catggtcaga agccgggcac atcagcgcct ggcagcagtg gcgtctggcg 3360
gaaaacctca gtgtgacgct ccccgccgcg tcccacgcca tcccgcatct gaccaccagc 3420
gaaatggatt tttgcatcga gctgggtaat aagcgttggc aatttaaccg ccagtcaggc 3480
tttctttcac agatgtggat tggcgataaa aaacaactgc tgacgccgct gcgcgatcag 3540
ttcacccgtg caccgctgga taacgacatt ggcgtaagtg aagcgacccg cattgaccct 3600
aacgcctggg tcgaacgctg gaaggcggcg ggccattacc aggccgaagc agcgttgttg 3660
cagtgcacgg cagatacact tgctgatgcg gtgctgatta cgaccgctca cgcgtggcag 3720
catcagggga aaaccttatt tatcagccgg aaaacctacc ggattgatgg tagtggtcaa 3780
atggcgatta ccgttgatgt tgaagtggcg agcgatacac cgcatccggc gcggattggc 3840
ctgaactgcc agctggcgca ggtagcagag cgggtaaact ggctcggatt agggccgcaa 3900
gaaaactatc ccgaccgcct tactgccgcc tgttttgacc gctgggatct gccattgtca 3960
gacatgtata ccccgtacgt cttcccgagc gaaaacggtc tgcgctgcgg gacgcgcgaa 4020
ttgaattatg gcccacacca gtggcgcggc gacttccagt tcaacatcag ccgctacagt 4080
caacagcaac tgatggaaac cagccatcgc catctgctgc acgcggaaga aggcacatgg 4140
ctgaatatcg acggtttcca tatggggatt ggtggcgacg actcctggag cccgtcagta 4200
tcggcggaat tccagctgag cgccggtcgc taccattacc agttggtctg gtgtcaaaaa 4260
taataataac cgggcaggcc atgtctgccc gtatttcgcg taaggaaatc cattatgtac 4320
tatttaaaaa acacaaactt ttggatgttc ggtttattct ttttctttta ctttttatc 4380
atgggagcct acttcccgtt tttcccgatt tggctacatg acatcaacca tatcagcaaa 4440
agtgatacgg gtattatttt tgccgctatt tctctgttct cgctattatt ccaaccgctg 4500
tttggtctgc tttctgacaa actcggaact tgtttattgc agcttataat ggttacaaat 4560
aaagcaatag catcacaaat ttcacaaata aagcattttt ttcactgcat tctagttgtg 4620
gtttgtccaa actcatcaat gtatcttatc atgtctggat ccagatctgg gcgtggctta 4680
agggtgggaa agaatatata aggtgggggt cttatgtagt tttgtatctg ttttgcagca 4740
gccgccgccg ccatgagcac caactcgttt gatggaagca ttgtgagctc atatttgaca 4800
acgcgcatgc ccccatgggc cggggtgcgt cagaatgtga tgggctccag cattgatggt 4860
cgccccgtcc tgcccgcaaa ctctactacc ttgacctacg agaccgtgtc tggaacgccg 4920
ttggagactg cagcctccgc cgccgcttca gccgctgcag ccaccgcccg cgggattgtg 4980
actgactttg ctttcctgag cccgcttgca agcagtgcag cttcccgttc atccgcccgc 5040
gatgacaagt tgacggctct tttggcacaa ttggattctt tgacccggga acttaatgtc 5100
gtttctcagc agctgttgga tctgcgccag caggtttctg ccctgaaggc ttcctcccct 5160
cccaatgcgg tttaaaacat aaataaaaaa ccagactctg tttggatttg gatcaagcaa 5220
gtgtcttgct gtctttattt aggggttttg cgcgcgcggt aggcccggga ccagcggtct 5280
cggtcgttga gggtcctgtg tatttttcc aggacgtggt aaaggtgact ctggatgttc 5340
agatacatgg gcataagccc gtctctgggg tggaggtagc accactgcag agcttcatgc 5400
tgcggggtgg tgttgtagat gatccagtcg tagcaggagc gctgggcgtg gtgcctaaaa 5460
atgtctttca gtagcaagct gattgccagg ggcaggccct tggtgtaagt gtttacaaag 5520
cggttaagct gggatgggtg catacgtggg gatatgagat gcatcttgga ctgtattttt 5580
aggttggcta tgttcccagc catatccctc cggggattca tgttgtgcag aaccaccagc 5640
acagtgtatc cggtgcactt gggaaatttg tcatgtagct tagaaggaaa tgcgtggaag 5700
aacttggaga cgcccttgtg acctccaaga ttttccatgc attcgtccat aatgatggca 5760
atgggcccac gggcggcggc ctgggcgaag atatttctgg gatcactaac gtcatagttg 5820
tgttccagga tgagatcgtc ataggccatt tttacaaagc gcgggcggag ggtgccagac 5880
tgcggtataa tggttccatc cggcccaggg gcgtagttac cctcacagat ttgcatttcc 5940
cacgctttga gttcagatgg ggggatcatg tctacctgcg gggcgatgaa gaaaacggtt 6000
tccgggtag gggagatcag ctgggaagaa agcaggttcc tgagcagctg cgacttaccg 6060
cagccggtgg gcccgtaaat cacacctatt accgggtgca actggtagtt aagagagctg 6120
cagctgccgt catccctgag caggggggcc acttcgttaa gcatgtccct gactcgcatg 6180
ttttccctga ccaaatccgc cagaaggcgc tcgccgccca gcgatagcag ttcttgcaag 6240
gaagcaaagt ttttcaacgg tttgagaccg tccgccgtag gcatgctttt gagcgtttga 6300
ccaagcagtt ccaggcggtc ccacagctcg gtcacctgct ctacggcatc tcgatccagc 6360
atatctcctc gtttcgcggg ttggggcggc tttcgctgta cggcagtagt cggtgctcgt 6420
ccagacgggc cagggtcatg tctttccacg ggcgcagggt cctcgtcagc gtagtctggg 6480
```

```
tcacggtgaa ggggtgcgct ccgggctgcg cgctggccag ggtgcgcttg aggctggtcc 6540
tgctggtgct gaagcgctgc cggtcttcgc cctgcgcgtc ggccaggtag catttgacca 6600
tggtgtcata gtccagcccc tccgcggcgt ggcccttggc gcgcagcttg cccttggagg 6660
aggcgccgca cgagggggcag tgcagacttt tgagggcgta gagcttgggc gcgagaaata 6720
ccgattccgg ggagtaggca tccgcgccgc aggcccccgca gacggtctcg cattccacga 6780
gccaggtgag ctctggccgt tcggggtcaa aaaccaggtt tcccccatgc tttttgatgc 6840
gtttcttacc tctggtttcc atgagccggt gtccacgctc ggtgacgaaa aggctgtccg 6900
tgtccccgta tacagacttg agaggcctgt cctcgagcgg tgttccgcgg tcctcctcgt 6960
atagaaactc ggaccactct gagacaaagg ctcgcgtcca ggccagcacg aaggaggcta 7020
agtgggaggg gtagcggtcg ttgtccacta gggggtccac tcgctccagg gtgtgaagac 7080
acatgtcgcc ctcttcggca tcaaggaagg tgattggttt gtaggtgtag gccacgtgac 7140
cgggtgttcc tgaagggggg ctataaaagg gggtgggggc gcgttcgtcc tcactctctt 7200
ccgcatcgct gtctgcgagg gccagctgtt ggggtgagta ctccctctga aaagcgggca 7260
tgacttctgc gctaagattg tcagtttcca aaaacgagga ggatttgata ttcacctggc 7320
ccgcggtgat gcctttgagg gtggccgcat ccatctggtc agaaaagaca atctttttgt 7380
tgtcaagctt ggtggcaaac gacccgtaga gggcgttgga cagcaacttg gcgatggagc 7440
gcagggtttg gtttttgtcg cgatcggcgc gctccttggc cgcgatgttt agctgcacgt 7500
attcgcgcgc aacgcaccgc cattcgggaa agacggtggt gcgctcgtcg ggcaccaggt 7560
gcacgcgcca accgcggttg tgcagggtga caaggtcaac gctggtggct acctctccgc 7620
gtaggcgctc gttggtccag cagaggcggc cgcccttgcg cgagcagaat ggcggtaggg 7680
ggtctagctg cgtctcgtcc ggggggtctg cgtccacggt aaagaccccg ggcagcaggc 7740
gcgcgtcgaa gtagtctatc ttgcatcctt gcaagtctag cgcctgctgc catgcgcggg 7800
cggcaagcgc gcgctcgtat gggttgagtg ggggaccccca tggcatgggg tgggtgagcg 7860
cggaggcgta catgccgcaa atgtcgtaaa cgtagagggg ctctctgagt attccaagat 7920
atgtagggta gcatcttcca ccgcggatgc tggcgcgcac gtaatcgtat agttcgtgcg 7980
agggagcgag gaggtcggga ccgaggttgc tacgggcggg ctgctctgct cggaagacta 8040
tctgcctgaa gatggcatgt gagttggatg atatggttgg acgctggaag acgttgaagc 8100
tggcgtctgt gagacctacc gcgtcacgca cgaaggaggc gtaggagtcg cgcagcttgt 8160
tgaccagctc ggcggtgacc tgcacgtcta gggcgcagta gtccagggtt tccttgatga 8220
tgtcatactt atcctgtccc ttttttttcc acagctcgcg gttgaggaca aactcttcgc 8280
ggtctttcca gtactcttgg atcggaaacc cgtcggcctc cgaacggtaa gagcctagca 8340
tgtagaactg gttgacggcc tggtaggcgc agcatccctt ttctacgggt agcgcgtatg 8400
cctgcgcggc cttccggagc gaggtgtggg tgagcgcaaa ggtgtccctg accatgactt 8460
tgaggtactg gtatttgaag tcagtgtcgt cgcatccgcc ctgctcccag agcaaaaagt 8520
ccgtgcgctt tttggaacgc ggatttggca gggcgaaggt gacatcgttg aagagtatct 8580
ttcccgcgcg aggcataaag ttgcgtgtga tgcggaaggg tcccggcacc tcggaacggt 8640
tgttaattac ctgggcggcg agcacgatct cgtcaaagcc gttgatgttg tggcccacaa 8700
tgtaaagttc caagaagcgc gggatgccct tgatggaagg caatttttta agttcctcgt 8760
aggtgagctc ttcaggggag ctgagcccgt gctctgaaag ggcccagtct gcaagatgag 8820
ggttggaagc gacgaatgag ctccacaggt cacgggccat tagcatttgc aggtggtcgc 8880
gaaaggtcct aaactggcga cctatggcca tttttctgg ggtgatgcag tagaaggtaa 8940
gcgggtcttg ttcccagcgg tcccatccaa ggttcgcggc taggtctcgc gcggcagtca 9000
ctagaggctc atctccgccg aacttcatga ccagcatgaa gggcacgagc tgcttcccaa 9060
aggcccccat ccaagtatag gtctctacat cgtaggtgac aaagagacgc tcggtgcgag 9120
gatgcgagcc gatcgggaag aactggatct cccgccacca attggaggag tggctattga 9180
tgtggtgaaa gtagaagtcc ctgcgacggg ccgaacactc gtgctggctt ttgtaaaaac 9240
gtgcgcagta ctggcagcgg tgcacgggct gtacatcctg cacgaggttg acctgacgac 9300
cgcgcacaag gaagcagagt gggaatttga gcccctcgcc tggcgggttt ggctggtggt 9360
cttctacttc ggctgcttgt ccttgaccgt ctggctgctc gaggggagtt acggtggatc 9420
ggaccaccac gccgcgcgag cccaaagtcc agatgtccgc gcgcggcggt cggagcttga 9480
tgacaacatc gcgcagatgg gagctgtcca tggtctggag ctcccgcggc gtcaggtcag 9540
gcgggagctc ctgcaggttt acctcgcata gacgggtcag ggcgcgggct agatccaggt 9600
gatacctaat ttccaggggc tggttggtgg cggcgtcgat ggcttgcaag aggccgcatc 9660
cccgcggcgc gactacggta ccgcgcggcg ggcggtgggc cgcgggggtg tccttggatg 9720
atgcatctaa aagcggtgac gcgggcgagc cccggaggt aggggggct ccggacccgc 9780
cgggagaggg ggcaggggca cgtcggcgcc gcgcgcgggc aggagctggt gctgcgcgcg 9840
taggttgctg gcgaacgcga cgacgcggcg gttgatctcc tgaatctggc gcctctgcgt 9900
gaagacgacg ggcccggtga gcttgagcct gaaagagagt tcgacagaat caatttcggt 9960
gtcgttgacg gcggcctggc gcaaaatctc ctgcacgtct cctgagttgt cttgataggc 10020
gatctcggcc atgaactgct cgatctcttc ctcctggaga tctccgcgtc cggctcgctc 10080
cacggtggcg gcgaggtcgt tggaaatgcg ggccatgagc tgcgagaagg cgttgaggcc 10140
tccctcgttc cagacgcggc tgtagaccac gccccttcg gcatcgcggg cgcgcatgac 10200
```

cacctgcgcg agattgagct ccacgtgccg ggcgaagacg gcgtagtttc gcaggcgctg 10260
aaagaggtag ttgagggtgg tggcggtgtg ttctgccacg aagaagtaca taacccagcg 10320
tcgcaacgtg gattcgttga tatccccccaa ggcctcaagg cgctccatgg cctcgtagaa 10380
gtccacggcg aagttgaaaa actgggagtt gcgcgccgac acggttaact cctcctccag 10440
aagacggatg agctcggcga cagtgtcgcg cacctcgcgc tcaaaggcta caggggcctc 10500
ttcttcttct tcaatctcct cttccataag ggcctcccct tcttcttctt ctggcggcgg 10560
tgggggaggg gggacacggc ggcgacgacg gcgcaccggg aggcggtcga caaagcgctc 10620
gatcatctcc ccgcggcgac ggcgcatggt ctcggtgacg gcgcggccgt tctcgcgggg 10680
gcgcagttgg aagacgccgc ccgtcatgtc ccggttatgg gttggcgggg ggctgccatg 10740
cggcagggat acggcgctaa cgatgcatct caacaattgt tgtgtaggta ctccgccgcc 10800
gagggacctg agcgagtccg catcgaccgg atcggaaaac ctctcgagaa aggcgtctaa 10860
ccagtcacag tcgcaaggta ggctgagcac cgtggcgggc ggcagcgggc ggcggtcggg 10920
gttgtttctg gcggaggtgc tgctgatgat gtaattaaag taggcggtct tgagacggcg 10980
gatggtcgac agaagcacca tgtccttggg tccggcctgc tgaatgcgca ggcggtcggc 11040
catgccccag gcttcgtttt gacatcggcg caggtctttg tagtagtctt gcatgagcct 11100
ttctaccggc acttcttctt ctccttcctc ttgtcctgca tctcttgcat ctatcgctgc 11160
ggcggcggcg gagtttggcc gtaggtggcg ccctcttcct cccatgcgtg tgaccccgaa 11220
gcccctcatc ggctgaagca gggctaggtc ggcgacaacg cgctcggcta atatggcctg 11280
ctgcacctgc gtgagggtag actggaagtc atccatgtcc acaaagcggt ggtatgcgcc 11340
cgtgttgatg gtgtaagtgc agttggccat aacggaccag ttaacggtct ggtgacccgg 11400
ctgcgagagc tcggtgtacc tgagacgcga gtaagccctc gagtcaaata cgtagtcgtt 11460
gcaagtccgc accaggtact ggtatcccac caaaaagtgc ggcggcggct ggcggtagag 11520
gggccagcgt agggtggccg gggctccggg ggcgagatct tccaacataa ggcgatgata 11580
tccgtagatg tacctggaca tccaggtgat gccggcggcg gtggtggagg cgcgcggaaa 11640
gtcgcggacg cggttccaga tgttgcgcag cggcaaaaag tgctccatgg tcgggacgct 11700
ctggccggtc aggcgcgcgc aatcgttgac gctctagacc gtgcaaaagg agagcctgta 11760
agcgggcact cttccgtggt ctggtggata aattcgcaag ggtatcatgg cggacgaccg 11820
gggttcgagc cccgtatccg gccgtccgcc gtgatccatg cggttaccgc ccgcgtgtcg 11880
aacccaggtg tgcgacgtca gacaacgggg gagtgctcct tttggcttcc ttccaggcgc 11940
ggcggctgct gcgctagctt ttttggccac tggccgcgcg cagcgtaagc ggttaggctg 12000
gaaagcgaaa gcattaagtg gctcgctccc tgtagccgga gggttatttt ccaagggttg 12060
agtcgcggga cccccggttc gagtctcgga ccggccggac tgcggcgaac gggggtttgc 12120
ctccccgtca tgcaagaccc cgcttgcaaa ttcctccgga aacagggacg agcccctttt 12180
ttgcttttcc cagatgcatc cggtgctgcg gcagatgcgc ccccctcctc agcagcggca 12240
agagcaagag cagcggcaga catgcagggc accctcccct cctcctaccg cgtcaggagg 12300
ggcgacatcc gcggttgacg cggcagcaga tggtgattac gaacccccgc ggcgccgggc 12360
ccggcactac ctggacttgg aggagggcga gggcctggcg cggctaggag cgccctctcc 12420
tgagcggtac ccaagggtgc agctgaagcg tgatacgcgt gaggcgtacg tgccgcggca 12480
gaacctgttt cgcgaccgcg agggagagga gcccgaggag atgcgggatc gaaagttcca 12540
cgcagggcgc gagctgcggc atggcctgaa tcgcgagcgg ttgctgcgcg aggaggactt 12600
tgagcccgac gcgcgaaccg ggattagtcc cgcgcgcgca cacgtggcgg ccgccgacct 12660
ggtaaccgca tacgagcaga cggtgaacca ggagattaac tttcaaaaaa gctttaacaa 12720
ccacgtgcgt acgcttgtgg cgcgcgagga ggtggctata ggactgatgc atctgtggga 12780
ctttgtaagc gcgctggagc aaaacccaaa tagcaagccg ctcatggcgc agctgttcct 12840
tatagtgcag cacagcaggg acaacgaggc attcagggat gcgctgctaa acatagtaga 12900
gcccgagggc cgctggctgc tcgatttgat aaacatcctg cagagcatag tggtgcagga 12960
gcgcagcttg agcctggctg acaaggtggc cgccatcaac tattccatgc ttagcctggg 13020
caagttttac gcccgcaaga tataccatac cccttacgtt cccatagaca aggaggtaaa 13080
gatcgagggg ttctacatgc gcatggcgct gaaggtgctt accttgagcg acgacctggg 13140
cgtttatcgc aacgagcgca tccacaaggc cgtgagcgtg agccggcggc gcgagctcag 13200
cgaccgcgag ctgatgcaca gcctgcaaag ggccctggct ggcacgggca gcggcgatag 13260
agaggccgag tcctactttg acgcgggcgc tgacctgcgc tgggccccaa gccgacgcgc 13320
cctggaggca gctggggccg gacctgggct ggcggtggca cccgcgcgcg ctggcaacgt 13380
cggcggcgtg gaggaatatg acgaggacga tgagtacgag ccagaggacg gcgagtacta 13440
agcggtgatg tttctgatca gatgatgcaa gacgcaacgg acccggcggt gcgggcggcg 13500
ctgcagagcc agccgtccgg ccttaactcc acggacgact ggcgccaggt catggaccgc 13560
atcatgtcgc tgactgcgcg caatcctgac gcgttccggc agcagccgca ggccaaccgg 13620
ctctccgcaa ttctggaagc ggtggtcccg gcgcgcgcaa accccacgca cgagaaggtg 13680
ctggcgatcg taaacgcgct ggccgaaaac agggccatcc ggcccgacga ggccggcctg 13740
gtctacgacg cgctgcttca gcgcgtggct cgttacaaca gcggcaacgt gcagaccaac 13800
ctggaccggc tggtggggga tgtgcgcgag gccgtggcgc agcgtgagcg cgcgcagcag 13860
cagggcaacc tgggctccat ggttgcacta aacgccttcc tgagtacaca gcccgccaac 13920

gtgccgcggg gacaggagga ctacaccaac tttgtgagcg cactgcggct aatggtgact 13980
gagacaccgc aaagtgaggt gtaccagtct gggccagact atttttttcca gaccagtaga 14040
caaggcctgc agaccgtaaa cctgagccag gctttcaaaa acttgcaggg gctgtggggg 14100
gtgcgggctc ccacaggcga ccgcgcgacc gtgtctagct tgctgacgcc caactcgcgc 14160
ctgttgctgc tgctaatagc gcccttcacg gacagtggca gcgtgtcccg ggacacatac 14220
ctaggtcact tgctgacact gtaccgcgag gccataggtc aggcgcatgt ggacgagcat 14280
actttccagg agattacaag tgtcagccgc gcgctggggc aggaggacac gggcagcctg 14340
gaggcaaccc taaactacct gctgaccaac cggcggcaga agatcccctc gttgcacagt 14400
ttaaacagcg aggaggagcg catttgcgc tacgtgcagc agagcgtgag ccttaacctg 14460
atgcgcgacg gggtaacgcc cagcgtggcg ctggacatga ccgcgcgcaa catggaaccg 14520
ggcatgtatg cctcaaaccg gccgtttatc aaccgcctaa tggactactt gcatcgcgcg 14580
gccgccgtga accccgagta tttcaccaat gccatcttga acccgcactg gctaccgccc 14640
cctggtttct acaccggggg attcgaggtg cccgagggta acgatggatt cctctgggac 14700
gacatagacg acagcgtgtt ttccccgcaa ccgcagaccc tgctagagtt gcaacagcgc 14760
gagcaggcag aggcggcgct gcgaaaggaa agcttccgca ggccaagcag cttgtccgat 14820
ctaggcgctg cggccccgcg gtcagatgct agtagcccat ttccaagctt gatagggtct 14880
cttaccagca ctcgcaccac ccgcccgcgc ctgctgggcg aggaggagta cctaaacaac 14940
tcgctgctgc agccgcagcg cgaaaaaaac ctgcctccgg catttcccaa caacgggata 15000
gagagcctag tggacaagat gagtagatgg aagacgtacg cgcaggagca cagggacgtg 15060
ccaggcccgc gcccgcccac ccgtcgtcaa aggcacgacc gtcagcgggg tctggtgtgg 15120
gaggacgatg actcggcaga cgacagcagc gtcctggatt tgggagggag tggcaacccg 15180
tttgcgcacc ttcgccccag gctggggaga atgttttaaa aaaaaaaaag catgatgcaa 15240
aataaaaaac tcaccaaggc catggcaccg agcgttggtt ttcttgtatt ccccttagta 15300
tgcggcgcgc ggcgatgtat gaggaaggtc ctcctccctc ctacgagagt gtggtgagcg 15360
cggcgccagt ggcggcggcg ctgggttctc ccttcgatgc tcccctggac ccgccgtttg 15420
tgcctccgcg gtacctgcgg cctaccgggg ggagaaacag catccgttac tctgagttgg 15480
cacccctatt cgacaccacc cgtgtgtacc tggtggacaa caagtcaacg gatgtggcat 15540
ccctgaacta ccagaacgac cacagcaact ttctgaccac ggtcattcaa aacaatgact 15600
acagcccggg ggaggcaagc acacagacca tcaatcttga cgaccggtcg cactggggcg 15660
gcgacctgaa aaccatcctg cataccaaca tgccaaatgt gaacgagttc atgtttacca 15720
ataagtttaa ggcgcgggtg atggtgtcgc gcttgcctac taaggacaat caggtggagc 15780
tgaaatacga gtgggtggag ttcacgctgc ccgagggcaa ctactccgag accatgacca 15840
tagaccttat gaacaacgcg atcgtggagc actacttgaa agtgggcaga cagaacgggg 15900
ttctggaaag cgacatcggg gtaaagtttg acacccgcaa cttcagactg gggtttgacc 15960
ccgtcactgg tcttgtcatg cctggggtat atacaaacga agccttccat ccagacatca 16020
ttttgctgcc aggatgcggg gtggacttca cccacagccg cctgagcaac ttgttgggca 16080
tccgcaagcg gcaacccttc caggagggct ttaggatcac ctacgatgat ctggagggtg 16140
gtaacattcc cgcactgttg gatgtggacg cctaccaggc gagcttgaaa gatgacaccg 16200
aacagggcgg gggtggcgca ggcggcagca acagcagtgg cagcggcgcg gaagagaact 16260
ccaacgcggc agccgcggca atgcagccgg tggaggacat gaacgatcat gccattcgcg 16320
gcgacacctt tgccacacgg gctgaggaga agcgcgctga ggccgaagca gcggccgaag 16380
ctgccgcccc cgctgcgcaa cccgaggtcg agaagcctca gaagaaaccg gtgatcaaac 16440
ccctgacaga ggacagcaag aaacgcagtt acaacctaat aagcaatgac agcaccttca 16500
cccagtaccg cagctggtac cttgcataca actacggcga ccctcagacc ggaatccgct 16560
catggaccct gctttgcact cctgacgtaa cctgcggctc ggagcaggtc tactggtcgt 16620
tgccagacat gatgcaagac cccgtgacct tccgctccac gcgccagatc agcaactttc 16680
cggtggtggg cgccgagctg ttgcccgtgc actccaagag cttctacaac gaccaggccg 16740
tctactccca actcatccgc cagtttacct ctctgaccca cgtgttcaat cgctttcccg 16800
agaaccagat tttggcgcgc ccgccagccc ccaccatcac caccgtcagt gaaaacgttc 16860
ctgctctcac agatcacggg acgctaccgc tgcgcaacag catcggagga gtccagcgag 16920
tgaccattac tgacgccaga cgccgcacct gcccctacgt ttacaaggcc ctgggcatag 16980
tctcgccgcg cgtcctatcg agccgcactt tttgagcaag catgtccatc cttatatcgc 17040
ccagcaataa cacaggctgg ggcctgcgct tcccaagcaa gatgtttggc ggggccaaga 17100
agcgctccga ccaacaccca gtgcgcgtgc gcgggcacta ccgcgcgccc tggggcgcgc 17160
acaaacgcgg ccgcactggg cgcaccaccg tcgatgacgc catcgacgcg gtggtggagg 17220
aggcgcgcaa ctacacgccc acgccgccac cagtgtccac agtggacgcg gccattcaga 17280
ccgtggtgcg cggagcccgg cgctatgcta aaatgaagag acggcggagg cgcgtagcac 17340
gtcgccaccg ccgccgaccc ggcactgccg cccaacgcgc ggcggcggcc ctgcttaacc 17400
gcgcacgtcg caccggccga cgggcggcca tgcgggccgc tcgaaggctg gccgcgggta 17460
ttgtcactgt gccccccagg tccaggcgac gagcggccgc cgcagcagcc gcggccatta 17520
gtgctatgac tcagggtcgc aggggcaacg tgtattgggt gcgcgactcg gttagcggcc 17580
tgcgcgtgcc cgtgcgcacc cgcccccccgc gcaactagat tgcaagaaaa aactacttag 17640

actcgtactg ttgtatgtat ccagcggcgg cggcgcgcaa cgaagctatg tccaagcgca 17700
aaatcaaaga agagatgctc caggtcatcg cgccggagat ctatggcccc ccgaagaagg 17760
aagagcagga ttacaagccc cgaaagctaa agcgggtcaa aaagaaaaag aaagatgatg 17820
atgatgaact tgacgacgag gtggaactgc tgcacgctac cgcgcccagg cgacgggtac 17880
agtggaaagg tcgacgcgta aaacgtgttt tgcgacccgg caccaccgta gtctttacgc 17940
ccggtgagcg ctccacccgc acctacaagc gcgtgtatga tgaggtgtac ggcgacgagg 18000
acctgcttga gcaggccaac gagcgcctcg ggagtttgc ctacggaaag cggcataagg 18060
acatgctggc gttgccgctg gacgagggca acccaacacc tagcctaaag cccgtaacac 18120
tgcagcaggt gctgcccgcg cttgcaccgt ccgaagaaaa gcgcggccta aagcgcgagt 18180
ctggtgactt ggcacccacc gtgcagctga tggtacccaa gcgccagcga ctggaagatg 18240
tcttggaaaa aatgaccgtg gaacctgggc tggagcccga ggtccgcgtg cggccaatca 18300
agcaggtggc gccgggactg ggcgtgcaga ccgtggacgt tcagataccc actaccagta 18360
gcaccagtat tgccaccgcc acagagggca tggagacaca aacgtccccg gttgcctcag 18420
cggtggcgga tgccgcggtg caggcggtcg ctgcggccgc gtccaagacc tctacggagg 18480
tgcaaacgga cccgtggatg tttcgcgttt cagcccccg gcgcccgcgc ggttcgagga 18540
agtacggcgc cgccagcgcg ctactgcccg aatatgccct acatccttcc attgcgccta 18600
cccccggcta tcgtggctac acctaccgcc ccagaagacg agcaactacc cgacgccgaa 18660
ccaccactgg aacccgccgc cgccgtcgcc gtcgccagcc cgtgctggcc ccgatttccg 18720
tgcgcagggt ggctcgcgaa ggaggcagga ccctggtgct gccaacagcg cgctaccacc 18780
ccagcatcgt ttaaaagccg gtctttgtgg ttcttgcaga tatggccctc acctgccgcc 18840
tccgtttccc ggtgccggga ttccgaggaa gaatgcaccg taggaggggc atggccggcc 18900
acggcctgac gggcggcatg cgtcgtgcgc accaccggcg gcggcgcgcg tcgcaccgtc 18960
gcatgcgcgg cggtatcctg cccctcctta ttccactgat cgccgcggcg attggcgccg 19020
tgcccggaat tgcatccgtg gccttgcagg cgcagagaca ctgattaaaa acaagttgca 19080
tgtggaaaaa tcaaaataaa aagtctggac tctcacgctc gcttggtcct gtaactattt 19140
tgtagaatgg aagacatcaa ctttgcgtct ctggccccgc gacacggctc gcgcccgttc 19200
atgggaaact ggcaagatat cggcaccagc aatatgagcg gtggcgcctt cagctggggc 19260
tcgctgtgga gcggcattaa aaatttcggt tccaccgtta agaactatgg cagcaaggcc 19320
tggaacagca gcacaggcca gatgctgagg gataagttga aagagcaaaa tttccaacaa 19380
aaggtggtag atggcctggc ctctggcatt agcggggtgg tggacctggc caaccaggca 19440
gtgcaaaata agattaacag taagcttgat ccccgccctc ccgtagagga gcctccaccg 19500
gccgtggaga cagtgtctcc agaggggcgt ggcgaaaagc gtccgcgccc cgacagggaa 19560
gaaactctgg tgacgcaaat agacgagcct ccctcgtacg aggaggcact aaagcaaggc 19620
ctgcccacca cccgtcccat cgcgcccatg gctaccggag tgctgggcca gcacacaccc 19680
gtaacgctgg acctgcctcc ccccgccgac acccagcaga aacctgtgct gccaggcccg 19740
accgccgttg ttgtaacccg tcctagccgc gcgtccctgc gccgcgccgc cagcggtccg 19800
cgatcgttgc ggcccgtagc cagtggcaac tggcaaagca cactgaacag catcgtgggt 19860
ctgggggtgc aatccctgaa gcgccgacga tgcttctgaa tagctaacgt gtcgtatgtg 19920
tgtcatgtat gcgtccatgt cgccgccaga ggagctgctg agccgccgcg cgcccgcttt 19980
ccaagatggc taccccttcg atgatgccgc agtggtctta catgcacatc tcgggccagg 20040
acgcctcgga gtacctgagc cccgggctgg tgcagtttgc ccgcgccacc gagacgtact 20100
tcagcctgaa taacaagttt agaaccccca cggtggcgcc tacgcacgac gtgaccacag 20160
accggtccca gcgtttgacg ctgcggttca tccctgtgga ccgtgaggat actgcgtact 20220
cgtacaaggc gcggttcacc ctagctgtgg gtgataaccg tgtgctggac atggcttcca 20280
cgtactttga catccgcggc gtgctggaca ggggccctac ttttaagccc tactctggca 20340
ctgcctacaa cgccctggct cccaagggtg ccccaaatcc ttgcgaatgg gatgaagctg 20400
ctactgctct tgaaataaac ctagaagaag aggacgatga caacgaagac gaagtagacg 20460
agcaagctga gcagcaaaaa actcacgtat ttgggcaggc gccttattct ggtataaata 20520
ttacaaagga gggtattcaa ataggtgtcg aaggtcaaac acctaaatat gccgataaaa 20580
catttcaacc tgaacctcaa ataggagaat ctcagtggta cgaaactgaa attaatcatg 20640
cagctgggag agtccttaaa aagactaccc caatgaaacc atgttacggt tcatatgcaa 20700
aacccacaaa tgaaaatgga gggcaaggca ttcttgtaaa gcaacaaaat ggaaagctag 20760
aaagtcaagt ggaaatgcaa tttttctcaa ctactgaggc gaccgcaggc aatggtgata 20820
acttgactcc taaagtggta ttgtacagtg aagatgtaga tatagaaacc ccagacactc 20880
atatttctta catgcccact attaaggaag gtaactcacg agaactaatg ggccaacaat 20940
ctatgcccaa caggcctaat tacattgctt ttagggacaa ttttattggt ctaatgtatt 21000
acaacagcac gggtaatatg ggtgttctgg cgggccaagc atcgcagttg aatgctgttg 21060
tagatttgca agacagaaac acagagcttt cataccagct tttgcttgat tccattggtg 21120
atagaaccag gtacttttct atgtggaatc aggctgttga cagctatgat ccagatgtta 21180
gaattattga aaatcatgga actgaagatg aacttccaaa ttactgcttt ccactgggag 21240
gtgtgattaa tacagagact cttaccaagg taaaacctaa aacaggtcag gaaaatggat 21300
gggaaaaaga tgctacagaa ttttcagata aaaatgaaat aagagttgga aataattttg 21360

ccatggaaat caatctaaat gccaacctgt ggagaaattt cctgtactcc aacatagcgc 21420
tgtatttgcc cgacaagcta aagtacagtc cttccaacgt aaaaatttct gataacccaa 21480
acacctacga ctacatgaac aagcgagtgg tggctcccgg gttagtggac tgctacatta 21540
accttggagc acgctggtcc cttgactata tggacaacgt caacccattt aaccaccacc 21600
gcaatgctgg cctgcgctac cgctcaatgt tgctgggcaa tggtcgctat gtgcccttcc 21660
acatccaggt gcctcagaag ttctttgcca ttaaaaacct ccttctcctg ccgggctcat 21720
acacctacga gtggaacttc aggaaggatg ttaacatggt tctgcagagc tccctaggaa 21780
atgacctaag ggttgacgga gccagcatta agtttgatag catttgcctt tacgccacct 21840
tcttccccat ggcccacaac accgcctcca cgcttgaggc catgcttaga aacgacacca 21900
acgaccagtc ctttaacgac tatctctccg ccgccaacat gctctaccct atacccgcca 21960
acgctaccaa cgtgcccata tccatcccct cccgcaactg ggcggctttc cgcggctggg 22020
ccttcacgcg ccttaagact aaggaaaccc catcactggg ctcgggctac gacccttatt 22080
acacctactc tggctctata ccctacctag atggaacctt ttacctcaac cacaccttta 22140
agaaggtggc cattaccttt gactcttctg tcagctggcc tggcaatgac cgcctgctta 22200
cccccaacga gtttgaaatt aagcgctcag ttgacgggga gggttacaac gttgcccagt 22260
gtaacatgac caaagactgg ttcctggtac aaatgctagc taactacaac attggctacc 22320
agggcttcta tatcccagag agctacaagg accgcatgta ctccttcttt agaaacttcc 22380
agcccatgag ccgtcaggtg gtggatgata ctaaatacaa ggactaccaa caggtgggca 22440
tcctacacca acacaacaac tctggatttg ttggctacct tgcccccacc atgcgcgaag 22500
gacaggccta ccctgctaac ttcccctatc cgcttatagg caagaccgca gttgacagca 22560
ttacccagaa aaagtttctt tgcgatcgca ccctttggcg catcccattc tccagtaact 22620
ttatgtccat gggcgcactc acagacctgg gccaaaacct tctctacgcc aactccgccc 22680
acgcgctaga catgactttt gaggtggatc ccatggacga gcccaccctt ctttatgttt 22740
tgtttgaagt ctttgacgtg gtccgtgtgc accggccgca ccgcggcgtc atcgaaaccg 22800
tgtacctgcg cacgcccttc tcggccggca acgccacaac ataaagaagc aagcaacatc 22860
aacaacagct gccgccatgg gctccagtga gcaggaactg aaagccattg tcaaagatct 22920
tggttgtggg ccatattttt tgggcaccta tgacaagcgc tttccaggct ttgtttctcc 22980
acacaagctc gcctgcgcca tagtcaatac ggccggtcgc gagactgggg gcgtacactg 23040
gatggccttt gcctggaacc cgcactcaaa aacatgctac ctctttgagc cctttggctt 23100
ttctgaccag cgactcaagc aggtttacca gtttgagtac gagtcactcc tgcgccgtag 23160
cgccattgct tcttcccccg accgctgtat aacgctggaa aagtccaccc aaagcgtaca 23220
ggggcccaac tcggccgcct gtggactatt ctgctgcatg tttctccacg cctttgccaa 23280
ctggccccaa actcccatgg atcacaaccc caccatgaac cttattaccg ggtacccaa 23340
ctccatgctc aacagtcccc aggtacagcc caccctgcgt cgcaaccagg aacagctcta 23400
cagcttcctg gagcgccact cgccctactt ccgcagccac agtgcgcaga ttaggagcgc 23460
cacttctttt tgtcacttga aaaacatgta aaaataatgt actagagaca ctttcaataa 23520
aggcaaatgc ttttatttgt acactctcgg gtgattattt acccccaccc ttgccgtctg 23580
cgccgtttaa aaatcaaagg ggttctgccg cgcatcgcta tgcgccactg gcagggacac 23640
gttgcgatac tggtgtttag tgctccactt aaactcaggc acaaccatcc gcggcagctc 23700
ggtgaagttt tcactccaca ggctgcgcac catcaccaac gcgtttagca ggtcgggcgc 23760
cgatatcttg aagtcgcagt tggggcctcc gccctgcgcg cgcgagttgc gatacacagg 23820
gttgcagcac tggaacacta tcagcgccgg gtggtgcacg ctggccagca cgctcttgtc 23880
ggagatcaga tccgcgtcca ggtcctccgc gttgctcagg gcgaacggag tcaactttgg 23940
tagctgcctt cccaaaaagg gcgcgtgccc aggctttgag ttgcactcgc accgtagtgg 24000
catcaaaagg tgaccgtgcc cggtctgggc gttaggatac agcgcctgca taaaagcctt 24060
gatctgctta aaagccacct gagcctttgc gccttcagag aagaacatgc cgcaagactt 24120
gccggaaaac tgattggccg gacaggccgc gtcgtgcacg cagcaccttg cgtcggtgtt 24180
ggagatctgc accacatttc ggccccaccg gttcttcacg atcttggcct tgctagactg 24240
ctccttcagc gcgcgctgcc cgttttcgct cgtcacatcc atttcaatca cgtgctcctt 24300
atttatcata atgcttccgt gtagacactt aagctcgcct tcgatctcag cgcagcggtg 24360
cagccacaac gcgcagcccg tgggctcgtg atgcttgtag gtcacctctg caaacgactg 24420
caggtacgcc tgcaggaatc gccccatcat cgtcacaaag gtcttgttgc tggtgaaggt 24480
cagctgcaac ccgcggtgct cctcgttcag ccaggtcttg catacggccg ccagagcttc 24540
cacttggtca ggcagtagtt tgaagttcgc ctttagatcg ttatccacgt ggtacttgtc 24600
catcagcgcg cgcgcagcct ccatgccctt ctcccacgca gacacgatcg gcacactcag 24660
cgggttcatc accgtaattt cactttccgc ttcgctgggc tcttcctctt cctcttgcgt 24720
ccgcatacca cgcgccactg ggtcgtcttc attcagccgc cgcactgtgc gcttacctcc 24780
tttgccatgc ttgattagca ccggtgggtt gctgaaaccc accatttgta gcgccacatc 24840
ttctctttct tcctcgctgt ccacgattac ctctggtgat ggcgggcgct cgggcttggg 24900
agaagggcgc ttcttttttct tcttgggcgc aatggccaaa tccgccgccg aggtcgatgg 24960
ccgcgggctg ggtgtgcgcg gcaccagcgc gtcttgtgat gagtcttcct cgtcctcgga 25020
ctcgatacgc cgcctcatcc gcttttttgg gggcgcccgg ggaggcggcg gcgacgggga 25080

cggggacgac acgtcctcca tggttggggg acgtcgcgcc gcaccgcgtc cgcgctcggg 25140
ggtggtttcg cgctgctcct cttcccgact ggccatttcc ttctcctata ggcagaaaaa 25200
gatcatggag tcagtcgaga agaaggacag cctaaccgcc ccctctgagt tcgccaccac 25260
cgcctccacc gatgccgcca acgcgcctac caccttcccc gtcgaggcac ccccgcttga 25320
ggaggaggaa gtgattatcg agcaggaccc aggttttgta agcgaagacg acgaggaccg 25380
ctcagtacca acagaggata aaaagcaaga ccaggacaac gcagaggcaa acgaggaaca 25440
agtcgggcgg ggggacgaaa ggcatggcga ctacctagat gtgggagacg acgtgctgtt 25500
gaagcatctg cagcgccagt gcgccattat ctgcgacgcg ttgcaagagc gcagcgatgt 25560
gcccctcgcc atagcggatg tcagccttgc ctacgaacgc cacctattct caccgcgcgt 25620
accccccaaa cgccaagaaa acggcacatg cgagcccaac ccgcgcctca acttctaccc 25680
cgtatttgcc gtgccagagg tgcttgccac ctatcacatc tttttccaaa actgcaagat 25740
acccctatcc tgccgtgcca accgcagccg agcggacaag cagctggcct tgcggcaggg 25800
cgctgtcata cctgatatcg cctcgctcaa cgaagtgcca aaaatctttg agggtcttgg 25860
acgcgacgag aagcgcgcgg caaacgctct gcaacaggaa aacagcgaaa atgaaagtca 25920
ctctggagtg ttggtggaac tcgagggtga caacgcgcgc ctagccgtac taaaacgcag 25980
catcgaggtc acccactttg cctacccggc acttaaccta ccccccaagg tcatgagcac 26040
agtcatgagt gagctgatcg tgcgccgtgc gcagcccctg gagagggatg caaatttgca 26100
agaacaaaca gaggagggcc tacccgcagt tggcgacgag cagctagcgc gctggcttca 26160
aacgcgcgag cctgccgact tggaggagcg acgcaaacta atgatggccg cagtgctcgt 26220
taccgtggag cttgagtgca tgcagcggtt ctttgctgac ccggagatgc agcgcaagct 26280
agaggaaaca ttgcactaca cctttcgaca gggctacgta cgccaggcct gcaagatctc 26340
caacgtggag ctctgcaacc tggtctccta ccttggaatt ttgcacgaaa accgccttgg 26400
gcaaaacgtg cttcattcca cgctcaaggg cgaggcgcgc cgcgactacg tccgcgactg 26460
cgtttactta tttctatgct acacctggca gacggccatg ggcgtttggc agcagtgctt 26520
ggaggagtgc aacctcaagg agctgcagaa actgctaaag caaaacttga aggacctatg 26580
gacggccttc aacgagcgct ccgtggccgc gcacctggcg gacatcattt tccccgaacg 26640
cctgcttaaa accctgcaac agggtctgcc agacttcacc agtcaaagca tgttgcagaa 26700
ctttaggaac tttatcctag agcgctcagg aatcttgccc gccacctgct gtgcacttcc 26760
tagcgacttt gtgcccatta agtaccgcga atgccctccg ccgctttggg gccactgcta 26820
ccttctgcag ctagccaact accttgccta ccactctgac ataatggaag acgtgagcgg 26880
tgacggtcta ctggagtgtc actgtcgctg caacctatgc accccgcacc gctccctggt 26940
ttgcaattcg cagctgctta acgaaagtca aattatcggt acctttgagc tgcagggtcc 27000
ctcgcctgac gaaaagtccg cggctccggg gttgaaactc actccggggc tgtggacgtc 27060
ggcttacctt cgcaaatttg tacctgagga ctaccacgcc cacgagatta ggttctacga 27120
agaccaatcc cgcccgccaa atgcggagct taccgcctgc gtcattaccc agggccacat 27180
tcttggccaa ttgcaagcca tcaacaaagc ccgccaagag tttctgctac gaaagggacg 27240
gggggtttac ttggaccccc agtccggcga ggagctcaac ccaatccccc cgccgccgca 27300
gccctatcag cagcagccgc gggcccttgc ttcccaggat ggcacccaaa aagaagctgc 27360
agctgccgcc gccacccacg gacgaggagg aatactggga cagtcaggca gaggaggttt 27420
tggacgagga ggaggaggac atgatggaag actgggagag cctagacgag gaagcttccg 27480
aggtcgaaga ggtgtcagac gaaacaccgt caccctcggt cgcattcccc tcgccggcgc 27540
cccagaaatc ggcaaccggt tccagcatgg ctacaacctc cgctcctcag gcgccgccgg 27600
cactgcccgt tcgccgaccc aaccgtagat gggacaccac tggaaccagg gccggtaagt 27660
ccaagcagcc gccgccgtta gcccaagagc aacaacagcg ccaaggctac cgctcatggc 27720
gcgggcacaa gaacgccata gttgcttgct tgcaagactg tgggggcaac atctccttcg 27780
cccgccgctt tcttctctac catcacggcg tggccttccc ccgtaacatc ctgcattact 27840
accgtcatct ctacagccca tactgcaccg gcggcagcgg cagcggcagc aacagcagcg 27900
gccacacaga agcaaaggcg accggatagc aagactctga caaagcccaa gaaatccaca 27960
gcggcggcag cagcaggagg aggagcgctg cgtctggcgc ccaacgaacc cgtatcgacc 28020
cgcgagctta gaaacaggat ttttcccact ctgtatgcta tatttcaaca gagcaggggc 28080
caagaacaag agctgaaaat aaaaaacagg tctctgcgat ccctcacccg cagctgcctg 28140
tatcacaaaa gcgaagatca gcttcggcgc acgctggaag acgcggaggc tctcttcagt 28200
aaatactgcg cgctgactct taaggactag tttcgcgccc tttctcaaat ttaagcgcga 28260
aaactacgtc atctccagcg gccacacccg gcgccagcac ctgtcgtcag cgccattatg 28320

```
ttcacgcctc gtcaggcaat cctaactctg cagacctcgt cctctgagcc gcgctctgga 28860
ggcattggaa ctctgcaatt tattgaggag tttgtgccat cggtctactt taacccсttc 28920
tcgggacctc ccggccacta tccggatcaa tttattccta actttgacgc ggtaaaggac 28980
tcggcggacg gctacgactg aatgttaagt ggagaggcag agcaactgcg cctgaaacac 29040
ctggtccact gtcgccgcca caagtgcttt gcccgcgact ccggtgagtt ttgctacttt 29100
gaattgcccg aggatcatat cgagggcccg gcgcacggcg tccggcttac cgcccaggga 29160
gagcttgccc gtagcctgat tcgggagttt acccagcgcc ccctgctagt tgagcgggac 29220
aggggaccct gtgttctcac tgtgatttgc aactgtccta accttggatt acatcaagat 29280
ttaattaatt gccacatcct cttacacttt ttcatacatt gcccaagaat aaagaatcgt 29340
ttgtgttatg tttcaacgtg tttatttttc aattgcagaa aatttcaagt catttttcat 29400
tcagtagtat agccccacca ccacatagct tatacagatc accgtacctt aatcaaactc 29460
acagaaccct agtattcaac ctgccacctc cctcccaaca cacagagtac acagtccttt 29520
ctccccggct ggccttaaaa agcatcatat catgggtaac agacatattc ttaggtgtta 29580
tattccacac ggtttcctgt cgagccaaac gctcatcagt gatattaata aactccccgg 29640
gcagctcact taagttcatg tcgctgtcca gctgctgagc cacaggctgc tgtccaactt 29700
gcggttgctt aacgggcggc gaaggagaag tccacgccta catgggggta gagtcataat 29760
cgtgcatcag gatagggcgg tggtgctgca gcagcgcgcg aataaactgc tgccgccgcc 29820
gctccgtcct gcaggaatac aacatggcag tggtctcctc agcgatgatt cgcaccgccc 29880
gcagcataag gcgccttgtc ctccgggcac agcagcgcac cctgatctca cttaaatcag 29940
cacagtaact gcagcacagc accacaatat tgttcaaaat cccacagtgc aaggcgctgt 30000
atccaaagct catggcgggg accacagaac ccacgtggcc atcataccac aagcgcaggt 30060
agattaagtg gcgacccctc ataaacacgc tggacataaa cattacctct tttggcatgt 30120
tgtaattcac cacctcccgg taccatataa acctctgatt aaacatggcg ccatccacca 30180
ccatcctaaa ccagctggcc aaaacctgcc cgccggctat acactgcagg gaaccgggac 30240
tggaacaatg acagtggaga gcccaggact cgtaaccatg gatcatcatg ctcgtcatga 30300
tatcaatgtt ggcacaacac aggcacacgt gcatacactt cctcaggatt acaagctcct 30360
cccgcgttag aaccatatcc cagggaacaa cccattcctg aatcagcgta aatcccacac 30420
tgcagggaag acctcgcacg taactcacgt tgtgcattgt caaagtgtta cattcgggca 30480
gcagcggatg atcctccagt atggtagcgc gggtttctgt ctcaaaagga ggtagacgat 30540
ccctactgta cggagtgcgc cgagacaacc gagatcgtgt tggtcgtagt gtcatgccaa 30600
atggaacgcc ggacgtagtc atatttcctg aagcaaaacc aggtgcgggc gtgacaaaca 30660
gatctgcgtc tccggtctcg ccgcttagat cgctctgtgt agtagttgta gtatatccac 30720
tctctcaaag catccaggcg ccccctggct tcgggttcta tgtaaactcc ttcatgcgcc 30780
gctgccctga taacatccac caccgcagaa taagccacac ccagccaacc tacacattcg 30840
ttctgcgagt cacacacggg aggagcggga agagctggaa gaaccatgtt ttttttttta 30900
ttccaaaaga ttatccaaaa cctcaaaatg aagatctatt aagtgaacgc gctcccctcc 30960
ggtggcgtgg tcaaactcta cagccaaaga acagataatg gcatttgtaa gatgttgcac 31020
aatggcttcc aaaaggcaaa cggccctcac gtccaagtgg acgtaaaggc taaacccttc 31080
agggtgaatc tcctctataa acattccagc accttcaacc atgcccaaat aattctcatc 31140
tcgccacctt ctcaatatat ctctaagcaa atcccgaata ttaagtccgg ccattgtaaa 31200
aatctgctcc agagcgccct ccaccttcag cctcaagcag cgaatcatga ttgcaaaaat 31260
tcaggttcct cacagacctg tataagattc aaaagcggaa cattaacaaa aataccgcga 31320
tccccgtaggt cccttcgcag ggccagctga acataatcgt gcaggtctgc acggaccagc 31380
gcggccactt ccccgccagg aaccttgaca aaagaaccca cactgattat gacacgcata 31440
ctcggagcta tgctaaccag cgtagccccg atgtaagctt tgttgcatgg gcggcgatat 31500
aaaatgcaag gtgctgctca aaaaatcagg caaagcctcg cgcaaaaaag aaagcacatc 31560
gtagtcatgc tcatgcagat aaaggcaggt aagctccgga accaccacag aaaaagacac 31620
cattttctc tcaaacatgt ctgcgggttt ctgcataaac acaaaataaa ataacaaaaa 31680
aacatttaaa cattagaagc ctgtcttaca acaggaaaaa caacccttat aagcataaga 31740
cggactacgg ccatgccggc gtgaccgtaa aaaaactggt caccgtgatt aaaaagcacc 31800
accgacagct cctcggtcat gtccggagtc ataatgtaag actcggtaaa cacatcaggt 31860
tgattcatcg gtcagtgcta aaaagcgacc gaaatagccc gggggaatac atacccgcag 31920
gcgtagagac aacattacag cccccatagg aggtataaca aaattaatag gagagaaaaa 31980
cacataaaca cctgaaaaac cctcctgcct aggcaaaata gcaccctccc gctccagaac 32040
aacatacagc gcttcacagc ggcagcctaa cagtcagcct taccagtaaa aaagaaaacc 32100
tattaaaaaa acaccactcg acacggcacc agctcaatca gtcacagtgt aaaaaagggc 32160
caagtgcaga gcgagtatat ataggactaa aaaatgacgt aacggttaaa gtccacaaaa 32220
aacacccaga aaaccgcacg cgaacctacg cccagaaacg aaagccaaaa aacccacaac 32280
ttcctcaaat cgtcacttcc gttttcccac gttacgtaac ttcccatttt aagaaaacta 32340
caattcccaa cacatacaag ttactccgcc ctaaaaccta cgtcacccgc cccgttccca 32400
cgccccgcgc cacgtcacaa actccacccc ctcattatca tattggcttc aatccaaaat 32460
aaggtatatt attgatgatg                                             32480
```

<210> 24
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 24
ctcaacaatt gtggatccgt actcc 25

<210> 25
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 25
gtgctcagca gatcttgcga ctgtg 25

<210> 26
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 26
ggcgcgttcg gatccactct cttcc 25

<210> 27
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 27
ctacatgcta ggcagatctc gttcggag 28

<210> 28
<211> 1240
<212> DNA
<213> adenovirus

<400> 28
ggatccactc tcttccgcat cgctgtctgc gagggccagc tgttggggtg agtactccct 60
ctgaaaagcg ggcatgactt ctgcgctaag attgtcagtt tccaaaaacg aggaggattt 120
gatattcacc tggcccgcgg tgatgccttt gagggtggcc gcatccatct ggtcagaaaa 180
gacaatcttt ttgttgtcaa gcttggtggc aaacgacccg tagagggcgt tggacagcaa 240
cttggcgatg gagcgcaggg tttggttttt gtcgcgatcg gcgcgctcct tggccgcgat 300
gtttagctgc acgtattcgc gcgcaacgca ccgccattcg ggaaagacgg tggtgcgctc 360
gtcgggcacc aggtgcacgc gccaaccgcg gttgtgcagg gtgacaaggt caacgctggt 420
ggctacctct ccgcgtaggc gctcgttggt ccagcagagg cggccgccct tgcgcgagca 480
gaatggcggt agggggtcta gctgcgtctc gtccgggggg tctgcgtcca cggtaaagac 540
cccgggcagc aggcgcgcgt cgaagtagtc tatcttgcat ccttgcaagt ctagcgcctg 600

```
ctgccatgcg cgggcggcaa gcgcgcgctc gtatgggttg agtgggggac cccatggcat 660
ggggtgggtg agcgcggagg cgtacatgcc gcaaatgtcg taaacgtaga ggggctctct 720
gagtattcca agatatgtag ggtagcatct tccaccgcgg atgctggcgc gcacgtaatc 780
gtatagttcg tgcgagggag cgaggaggtc gggaccgagg ttgctacggg cgggctgctc 840
tgctcggaag actatctgcc tgaagatggc atgtgagttg gatgatatgg ttggacgctg 900
gaagacgttg aagctggcgt ctgtgagacc taccgcgtca cgcacgaagg aggcgtagga 960
gtcgcgcagc ttgttgacca gctcggcggt gacctgcacg tctagggcgc agtagtccag 1020
ggtttccttg atgatgtcat acttatcctg tccctttttt ttccacagct cgcggttgag 1080
gacaaactct tcgcggtctt tccagtactc ttggatcgga aacccgtcgg cctccgaacg 1140
agatccgtac tccgccgccg agggacctga gcgagtccgc atcgaccgga tcggaaaacc 1200
tctcgagaaa ggcgtctaac cagtcacagt cgcaagatct                       1240
```

<210> 29
<211> 8383
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid pDV60

<400> 29

```
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gcttggtacc 900
gagctcggat ccactctctt ccgcatcgct gtctgcgagg gccagctgtt ggggtgagta 960
ctccctctga aaagcgggca tgacttctgc gctaagattg tcagtttcca aaaacgagga 1020
ggatttgata ttcacctggc ccgcggtgat gcctttgagg gtggccgcat ccatctggtc 1080
agaaaagaca atctttttgt tgtcaagctt ggtggcaaac gacccgtaga gggcgttgga 1140
cagcaacttg gcgatggagc gcagggtttg gtttttgtcg cgatcggcgc gctccttggc 1200
cgcgatgttt agctgcacgt attcgcgcgc aacgcaccgc cattcgggaa agacggtggt 1260
gcgctcgtcg ggcaccaggt gcacgcgcca accgcggttg tgcagggtga caaggtcaac 1320
gctggtggct acctctccgc gtaggcgctc gttggtccag cagaggcggc cgcccttgcg 1380
cgagcagaat ggcggtaggg ggtctagctg cgtctcgtcc ggggggtctg cgtccacggt 1440
aaagaccccg ggcagcaggc gcgcgtcgaa gtagtctatc ttgcatcctt gcaagtctag 1500
cgcctgctgc catgcgcggg cggcaagcgc gcgctcgtat gggttgagtg gggaccccca 1560
tggcatgggg tgggtgagcg cggaggcgta catgccgcaa atgtcgtaaa cgtagagggg 1620
ctctctgagt attccaagat atgtagggta gcatcttcca ccgcggatgc tggcgcgcac 1680
gtaatcgtat agttcgtgcg agggagcgag gaggtcggga ccgaggttgc tacgggcggg 1740
ctgctctgct cggaagacta tctgcctgaa gatggcatgt gagttggatg atatggttgg 1800
acgctggaag acgttgaagc tggcgtctgt gagacctacc gcgtcacgca cgaaggaggc 1860
gtaggagtcg cgcagcttgt tgaccagctc ggcggtgacc tgcacgtcta gggcgcagta 1920
gtccagggtt tccttgatga tgtcatactt atcctgtccc ttttttttcc acagctcgcg 1980
gttgaggaca aactcttcgc ggtctttcca gtactcttgg atcggaaacc cgtcggcctc 2040
cgaacgagat ccgtactccg ccgccgaggg acctgagcga gtccgcatcg accggatcgg 2100
aaaacctctc gagaaaggcg tctaaccagt cacagtcgca agatccaaga tgaagcgcgc 2160
aagaccgtct gaagatacct tcaacccccgt gtatccatat gacacggaaa ccggtcctcc 2220
aactgtgcct ttttcttactc ctccctttgt atcccccaat gggtttcaag agagtccccc 2280
tggggtactc tctttgcgcc tatccgaacc tctagttacc tccaatggca tgcttgcgct 2340
caaaatgggc aacggcctct ctctggacga ggccggcaac cttacctccc aaaatgtaac 2400
cactgtgagc ccacctctca aaaaaaccaa gtcaaacata aacctggaaa tatctgcacc 2460
```

cctcacagtt acctcagaag ccctaactgt ggctgccgcc gcacctctaa tggtcgcggg 2520
caacacactc accatgcaat cacaggcccc gctaaccgtg cacgactcca aacttagcat 2580
tgccacccaa ggacccctca cagtgtcaga aggaaagcta gccctgcaaa catcaggccc 2640
cctcaccacc accgatagca gtaccettac tatcactgcc tcacccccte taactactgc 2700
cactggtagc ttgggcattg acttgaaaga gcccatttat acacaaaatg gaaaactagg 2760
actaaagtac ggggctcctt tgcatgtaac agacgaccta aacactttga ccgtagcaac 2820
tggtccaggt gtgactatta ataatacttc cttgcaaact aaagttactg gagccttggg 2880
ttttgattca caaggcaata tgcaacttaa tgtagcagga ggactaagga ttgattctca 2940
aaacagacgc cttatacttg atgttagtta tccgtttgat gctcaaaacc aactaaatct 3000
aagactagga cagggccctc tttttataaa ctcagcccac aacttggata ttaactacaa 3060
caaaggcctt tacttgttta cagcttcaaa caattccaaa aagcttgagg ttaacctaag 3120
cactgccaag gggttgatgt ttgacgctac agccatagcc attaatgcag gagatgggct 3180
tgaatttggt tcacctaatg caccaaacac aaatcccctc aaaacaaaaa ttggccatgg 3240
cctagaattt gattcaaaca aggctatggt tcctaaacta ggaactggcc ttagttttga 3300
cagcacaggt gccattacag taggaaacaa aaataatgat aagctaactt tgtggaccac 3360
accagctcca tctcctaact gtagactaaa tgcagagaaa gatgctaaac tcactttggt 3420
cttaacaaaa tgtggcagtc aaatacttgc tacagtttca gttttggctg ttaaaggcag 3480
tttggctcca atatctggaa cagttcaaag tgctcatctt attataagat ttgacgaaaa 3540
tggagtgcta ctaaacaatt ccttcctgga cccagaatat tggaacttta gaaatggaga 3600
tcttactgaa ggcacagcct atacaaacgc tgttggattt atgcctaacc tatcagctta 3660
tccaaaatct cacggtaaaa ctgccaaaag taacattgtc agtcaagttt acttaaacgg 3720
agacaaaact aaacctgtaa cactaaccat tacactaaac ggtacacagg aaacaggaga 3780
cacaactcca agtgcatact ctatgtcatt ttcatgggac tggtctggcc acaactacat 3840
taatgaaata tttgccacat cctcttacac tttttcatac attgcccaag aataaaagaa 3900
gcggccgctc gagcatgcat ctagagggcc ctattctata gtgtcaccta aatgctagag 3960
ctcgctgatc agcctcgact gtgccttcta gttgccagcc atctgttgtt tgcccctccc 4020
ccgtgccttc cttgaccctg gaaggtgcca ctcccactgt cctttcctaa taaaatgagg 4080
aaattgcatc gcattgtctg agtaggtgtc attctattct ggggggtggg gtggggcagg 4140
acagcaaggg ggaggattgg gaagacaata gcaggcatgc tggggatgcg gtgggctcta 4200
tggcttctga ggcggaaaga accagctggg gctctagggg gtatccccac gcgccctgta 4260
gcggcgcatt aagcgcggcg ggtgtggtgg ttacgcgcag cgtgaccgct acacttgcca 4320
gcgccctagc gcccgctcct ttcgctttct tcccttcctt tctcgccacg ttcgccggct 4380
ttccccgtca agctctaaat cggggcatcc ctttagggtt ccgatttagt gctttacggc 4440
acctcgaccc caaaaaactt gattagggtg atggttcacg tagtgggcca tcgccctgat 4500
agacggtttt tcgccctttg acgttggagt ccacgttctt taatagtgga ctcttgttcc 4560
aaactggaac aacactcaac cctatctcgg tctattcttt tgatttataa gggattttgg 4620
ggatttcggc ctattggtta aaaaatgagc tgatttaaca aaaatttaac gcgaattaat 4680
tctgtggaat gtgtgtcagt tagggtgtgg aaagtcccca ggctccccag gcaggcagaa 4740
gtatgcaaag catgcatctc aattagtcag caaccaggtg tggaaagtcc ccaggctccc 4800
cagcaggcag aagtatgcaa agcatgcatc tcaattagtc agcaaccata gtcccgcccc 4860
taactccgcc catcccgccc ctaactccgc ccagttccgc ccattctccg ccccatggct 4920
gactaatttt ttttatttat gcagaggccg aggccgcctc tgcctctgag ctattccaga 4980
agtagtgagg aggctttttt ggaggcctag gcttttgcaa aaagctcccg ggagcttgta 5040
tatccatttt cggatctgat caagagacag gatgaggatc gtttcgcatg attgaacaag 5100
atggattgca cgcaggttct ccggccgctt gggtggagag gctattcggc tatgactggg 5160
cacaacagac aatcggctgc tctgatgccg ccgtgttccg gctgtcagcg caggggcgcc 5220
cggttctttt tgtcaagacc gacctgtccg gtgccctgaa tgaactgcag gacgaggcag 5280
cgcggctatc gtggctggcc acgacgggcg ttccttgcgc agctgtgctc gacgttgtca 5340
ctgaagcggg aagggactgg ctgctattgg gcgaagtgcc ggggcaggat ctcctgtcat 5400
ctcaccttgc tcctgccgag aaagtatcca tcatggctga tgcaatgcgg cggctgcata 5460
cgcttgatcc ggctacctgc ccattcgacc accaagcgaa acatcgcatc gagcgagcac 5520
gtactcggat ggaagccggt cttgtcgatc aggatgatct ggacgaagag catcaggggc 5580
tcgcgccagc cgaactgttc gccaggctca aggcgcgcat gcccgacggc gaggatctcg 5640
tcgtgaccca tggcgatgcc tgcttgccga atatcatggt ggaaaatggc cgcttttctg 5700
gattcatcga ctgtggccgg ctgggtgtgg cggaccgcta tcaggacata gcgttggcta 5760
cccgtgatat tgctgaagag cttggcggcg aatgggctga ccgcttcctc gtgctttacg 5820
gtatcgccgc tcccgattcg cagcgcatcg ccttctatcg ccttcttgac gagttcttct 5880
gagcgggact ctggggttcg aaatgaccga ccaagcgacg cccaacctgc catcacgaga 5940
tttcgattcc accgccgcct tctatgaaag gttgggcttc ggaatcgttt tccgggacgc 6000
cggctggatg atcctccagc gcggggatct catgctggag ttcttcgccc accccaactt 6060
gtttattgca gcttataatg gttacaaata aagcaatagc atcacaaatt tcacaaataa 6120
agcatttttt tcactgcatt ctagttgtgg tttgtccaaa ctcatcaatg tatcttatca 6180

tgtctgtata ccgtcgacct ctagctagag cttggcgtaa tcatggtcat agctgtttcc 6240
tgtgtgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa gcataaagtg 6300
taaagcctgg ggtgcctaat gagtgagcta actcacatta attgcgttgc gctcactgcc 6360
cgctttccag tcgggaaacc tgtcgtgcca gctgcattaa tgaatcggcc aacgcgcggg 6420
gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact cgctgcgctc 6480
ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac ggttatccac 6540
agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa 6600
ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg acgagcatca 6660
caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa gataccaggc 6720
gtttccccct ggaagctccc tcgtgcgctc tcctgttccg accctgccgc ttaccggata 6780
cctgtccgcc tttctccctt cgggaagcgt ggcgctttct caatgctcac gctgtaggta 6840
tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac cccccgttca 6900
gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg taagacacga 6960
cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt atgtaggcgg 7020
tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga cagtatttgg 7080
tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct cttgatccgg 7140
caaacaaacc accgctggta gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag 7200
aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg ctcagtggaa 7260
cgaaaactca cgttaaggga ttttggtcat gagattatca aaaaggatct tcacctagat 7320
ccttttaaat taaaaatgaa gttttaaatc aatctaaagt atatatgagt aaacttggtc 7380
tgacagttac caatgcttaa tcagtgaggc acctatctca gcgatctgtc tatttcgttc 7440
atccatagtt gcctgactcc ccgtcgtgta gataactacg atacgggagg gcttaccatc 7500
tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag atttatcagc 7560
aataaaccag ccagccggaa gggccgagcg cagaagtggt cctgcaactt tatccgcctc 7620
catccagtct attaattgtt gccgggaagc tagagtaagt agttcgccag ttaatagttt 7680
gcgcaacgtt gttgccattg ctacaggcat cgtggtgtca cgctcgtcgt ttggtatggc 7740
ttcattcagc tccggttccc aacgatcaag gcgagttaca tgatccccca tgttgtgcaa 7800
aaaagcggtt agctccttcg gtcctccgat cgttgtcaga agtaagttgg ccgcagtgtt 7860
atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat ccgtaagatg 7920
cttttctgtg actggtgagt actcaaccaa gtcattctga gaatagtgta tgcggcgacc 7980
gagttgctct tgcccggcgt caatacggga taataccgcg ccacatagca gaactttaaa 8040
agtgctcatc attggaaaac gttcttcggg gcgaaaactc tcaaggatct taccgctgtt 8100
gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat cttttacttt 8160
caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa agggaataag 8220
ggcgacacgg aaatgttgaa tactcatact cttccttttt caatattatt gaagcattta 8280
tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa ataaacaaat 8340
aggggttccg cgcacatttc cccgaaaagt gccacctgac gtc 8383

<210> 30
<211> 7960
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid pDV67

<400> 30
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc 900

gtttaaactt aagcttggta ccgagctcgg atccactctc ttccgcatcg ctgtctgcga 960
gggccagctg ttggggtgag tactccctct gaaaagcggg catgacttct gcgctaagat 1020
tgtcagtttc caaaaacgag gaggatttga tattcacctg gcccgcggtg atgcctttga 1080
gggtggccgc atccatctgg tcagaaaaga caatcttttt gttgtcaagc ttggtggcaa 1140
acgacccgta gagggcgttg gacagcaact tggcgatgga gcgcagggtt tggtttttgt 1200
cgcgatcggc gcgctccttg gccgcgatgt ttagctgcac gtattcgcgc gcaacgcacc 1260
gccattcggg aaagacggtg gtgcgctcgt cgggcaccag gtgcacgcgc caaccgcggt 1320
tgtgcagggt gacaaggtca acgctggtgg ctacctctcc gcgtaggcgc tcgttggtcc 1380
agcagaggcg gccgcccttg cgcgagcaga atggcggtag ggggtctagc tgcgtctcgt 1440
ccggggggtc tgcgtccacg gtaaagaccc cgggcagcag gcgcgcgtcg aagtagtcta 1500
tcttgcatcc ttgcaagtct agcgcctgct gccatgcgcg ggcggcaagc gcgcgctcgt 1560
atgggttgag tgggggaccc catggcatgg ggtgggtgag cgcggaggcg tacatgccgc 1620
aaatgtcgta aacgtagagg ggctctctga gtattccaag atatgtaggg tagcatcttc 1680
caccgcggat gctggcgcgc acgtaatcgt atagttcgtg cgagggagcg aggaggtcgg 1740
gaccgaggtt gctacgggcg ggctgctctg ctcggaagac tatctgcctg aagatggcat 1800
gtgagttgga tgatatggtt ggacgctgga agacgttgaa gctggcgtct gtgagaccta 1860
ccgcgtcacg cacgaaggag gcgtaggagt cgcgcagctt gttgaccagc tcggcggtga 1920
cctgcacgtc tagggcgcag tagtccaggg tttccttgat gatgtcatac ttatcctgtc 1980
cctttttttt ccacagctcg cggttgagga caaactcttc gcggtctttc cagtactctt 2040
ggatcggaaa cccgtcggcc tccgaacgag atccgtactc cgccgccgag ggacctgagc 2100
gagtccgcat cgaccggatc ggaaaacctc tcgagaaagg cgtctaacca gtcacagtcg 2160
caagatccaa gatgaagcgc gcaagaccgt ctgaagatac cttcaacccc gtgtatccat 2220
atgacacgga aaccggtcct ccaactgtgc cttttcttac tcctcccttt gtatccccca 2280
atgggtttca agagagtccc cctggggtac tctctttgcg cctatccgaa cctctagtta 2340
cctccaatgg catgcttgcg ctcaaaatgg gcaacggcct ctctctggac gaggccggca 2400
accttacctc ccaaaatgta accactgtga gcccacctct caaaaaaacc aagtcaaaca 2460
taaacctgga aatatctgca cccctcacag ttacctcaga agccctaact gtggctgccg 2520
ccgcacctct aatggtcgcg ggcaacacac tcaccatgca atcacaggcc ccgctaaccg 2580
tgcacgactc caaacttagc attgccaccc aaggacccct cacagtgtca gaaggaaagc 2640
tagccctgca aacatcaggc cccctcacca ccaccgatag cagtaccctt actatcactg 2700
cctcaccccc tctaactact gccactggta gcttgggcat tgacttgaaa gagcccattt 2760
atacacaaaa tggaaaacta ggactaaagt acggggctcc tttgcatgta acagacgacc 2820
taaacacttt gaccgtagca actggtccag gtgtgactat taataatact tccttgcaaa 2880
ctaaagttac tggagccttg ggttttgatt cacaaggcaa tatgcaactt aatgtagcag 2940
gaggactaag gattgattct caaaacagac gccttatact tgatgttagt tatccgtttg 3000
atgctcaaaa ccaactaaat ctaagactag gacagggccc tctttttata aactcagccc 3060
acaacttgga tattaactac aacaaaggcc tttacttgtt tacagcttca aacaattcca 3120
aaaagcttga ggttaaccta agcactgcca aggggttgat gtttgacgct acagccatag 3180
ccattaatgc aggagatggg cttgaatttg gttcacctaa tgcaccaaac acaaatcccc 3240
tcaaaacaaa aattggccat ggcctagaat ttgattcaaa caaggctatg gttcctaaac 3300
taggaactgg ccttagtttt gacagcacag gtgccattac agtaggaaac aaaaataatg 3360
ataagctaac tttgtggacc acaccagctc catctcctaa ctgtagacta aatgcagaga 3420
aagatgctaa actcactttg gtcttaacaa aatgtggcag tcaaatactt gctacagttt 3480
cagttttggc tgttaaaggc agtttggctc caatatctgg aacagttcaa agtgctcatc 3540
ttattataag atttgacgaa aatggagtgc tactaaacaa ttccttcctg gacccagaat 3600
attggaactt tagaaatgga gatcttactg aaggcacagc ctatacaaac gctgttggat 3660
ttatgcctaa cctatcagct tatccaaaat ctcacggtaa aactgccaaa agtaacattg 3720
tcagtcaagt ttacttaaac ggagacaaaa ctaaacctgt aacactaacc attacactaa 3780
acggtacaca ggaaacagga gacacaactc caagtgcata ctctatgtca ttttcatggg 3840
actggtctgg ccacaactac attaatgaaa tatttgccac atcctcttac actttttcat 3900
acattgccca agaataaaag aagcggccgc tcgagtctag agggcccgtt taaacccgct 3960
gatcagcctc gactgtgcct tctagttgcc agccatctgt tgtttgcccc tcccccgtgc 4020
cttccttgac cctggaaggt gccactccca ctgtcctttc ctaataaaat gaggaaattg 4080
catcgcattg tctgagtagg tgtcattcta ttctgggggg tggggtgggg caggacagca 4140
agggggagga ttgggaagac aatagcaggc atgctgggga tgcggtgggc tctatggctt 4200
ctgaggcgga aagaaccagc tggggctcta gggggtatcc ccacgcgccc tgtagcggcg 4260
cattaagcgc ggcgggtgtg gtggttacgc gcagcgtgac cgctacactt gccagcgccc 4320
tagcgcccgc tcctttcgct ttcttccctt cctttctcgc cacgttcgcc ggctttcccc 4380
gtcaagctct aaatcggggc atccctttag ggttccgatt tagtgcttta cggcacctcg 4440
accccaaaaa acttgattag ggtgatggtt cacgtagtgg gccatcgccc tgatagacgg 4500
tttttcgccc tttgacgttg gagtccacgt tctttaatag tggactcttg ttccaaactg 4560
gaacaacact caaccctatc tcggtctatt cttttgattt ataagggatt ttggggattt 4620

cggcctattg gttaaaaaat gagctgattt aacaaaaatt taacgcgaat taattctgtg 4680
gaatgtgtgt cagttagggt gtggaaagtc cccaggctcc ccaggcaggc agaagtatgc 4740
aaagcatgca tctcaattag tcagcaacca ggtgtggaaa gtccccaggc tccccagcag 4800
gcagaagtat gcaaagcatg catctcaatt agtcagcaac catagtcccg cccctaactc 4860
cgcccatccc gcccctaact ccgcccagtt ccgcccattc tccgccccat ggctgactaa 4920
ttttttttat ttatgcagag gccgaggccg cctctgcctc tgagctattc cagaagtagt 4980
gaggaggctt ttttggaggc ctaggctttt gcaaaaagct cccgggagct tgtatatcca 5040
ttttcggatc tgatcagcac gtgttgacaa ttaatcatcg gcatagtata tcggcatagt 5100
ataatacgac aaggtgagga actaaaccat ggccaagttg accagtgccg ttccggtgct 5160
caccgcgcgc gacgtcgccg gagcggtcga gttctggacc gaccggctcg ggttctcccg 5220
ggacttcgtg gaggacgact tcgccggtgt ggtccgggac gacgtgaccc tgttcatcag 5280
cgcggtccag gaccaggtgg tgccggacaa caccctggcc tgggtgtggg tgcgcggcct 5340
ggacgagctg tacgccgagt ggtcggaggt cgtgtccacg aacttccggg acgcctccgg 5400
gccggccatg accgagatcg gcgagcagcc gtgggggcgg gagttcgccc tgcgcgaccc 5460
ggccggcaac tgcgtgcact tcgtggccga ggagcaggac tgacacgtgc tacgagattt 5520
cgattccacc gccgccttct atgaaaggtt gggcttcgga atcgttttcc gggacgccgg 5580
ctggatgatc ctccagcgcg gggatctcat gctggagttc ttcgcccacc ccaacttgtt 5640
tattgcagct tataatggtt acaaataaag caatagcatc acaaatttca caaataaagc 5700
atttttttca ctgcattcta gttgtggttt gtccaaactc atcaatgtat cttatcatgt 5760
ctgtataccg tcgacctcta gctagagctt ggcgtaatca tggtcatagc tgtttcctgt 5820
gtgaaattgt tatccgctca caattccaca caacatacga gccggaagca taaagtgtaa 5880
agcctggggt gcctaatgag tgagctaact cacattaatt gcgttgcgct cactgcccgc 5940
tttccagtcg ggaaacctgt cgtgccagct gcattaatga atcggccaac gcgcggggag 6000
aggcggtttg cgtattgggc gctcttccgc ttcctcgctc actgactcgc tgcgctcggt 6060
cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga 6120
atcaggggat aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg ccaggaaccg 6180
taaaaaggcc gcgttgctgg cgtttttcca taggctccgc ccccctgacg agcatcacaa 6240
aaatcgacgc tcaagtcaga ggtggcgaaa cccgacagga ctataaagat accaggcgtt 6300
tccccctgga agctccctcg tgcgctctcc tgttccgacc ctgccgctta ccggatacct 6360
gtccgccttt ctcccttcgg gaagcgtggc gctttctcaa tgctcacgct gtaggtatct 6420
cagttcggtg taggtcgttc gctccaagct gggctgtgtg cacgaacccc ccgttcagcc 6480
cgaccgctgc gccttatccg gtaactatcg tcttgagtcc aacccggtaa gacacgactt 6540
atcgccactg gcagcagcca ctggtaacag gattagcaga gcgaggtatg taggcggtgc 6600
tacagagttc ttgaagtggt ggcctaacta cggctacact agaaggacag tatttggtat 6660
ctgcgctctg ctgaagccag ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa 6720
acaaaccacc gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa 6780
aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc agtggaacga 6840
aaactcacgt taagggattt tggtcatgag attatcaaaa aggatcttca cctagatcct 6900
tttaaattaa aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga 6960
cagttaccaa tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc 7020
catagttgcc tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg 7080
ccccagtgct gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat 7140
aaaccagcca gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat 7200
ccagtctatt aattgttgcc gggaagctag agtaagtagt tcgccagtta atagtttgcg 7260
caacgttgtt gccattgcta caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc 7320
attcagctcc ggttcccaac gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa 7380
agcggttagc tccttcggtc ctccgatcgt tgtcagaagt aagttggccg cagtgttatc 7440
actcatggtt atggcagcac tgcataattc tcttactgtc atgccatccg taagatgctt 7500
ttctgtgact ggtgagtact caaccaagtc attctgagaa tagtgtatgc ggcgaccgag 7560
ttgctcttgc ccggcgtcaa tacgggataa taccgcgcca catagcagaa ctttaaaagt 7620
gctcatcatt ggaaaacgtt cttcggggcg aaaactctca aggatcttac cgctgttgag 7680
atccagttcg atgtaaccca ctcgtgcacc caactgatct tcagcatctt ttactttcac 7740
cagcgtttct gggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc 7800
gacacggaaa tgttgaatac tcatactctt cctttttcaa tattattgaa gcatttatca 7860
gggttattgt ctcatgagcg gatacatatt tgaatgtatt tagaaaaata aacaaatagg 7920
ggttccgcgc acatttcccc gaaaagtgcc acctgacgtc 7960

<210> 31
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 31
atgggatcca agatgaagcg cgcaagaccg 30

<210> 32
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 32
cactatagcg gccgcattct cagtcatctt 30

<210> 33
<211> 7989
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid pDV69

<400> 33
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 300
tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc 360
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt 540
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 600
tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca 840
ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc 900
gtttaaactt aagcttggta ccgagctcgg atccactctc ttccgcatcg ctgtctgcga 960
gggccagctg ttggggtgag tactccctct gaaaagcggg catgacttct gcgctaagat 1020
tgtcagtttc caaaaacgag gaggatttga tattcacctg gcccgcggtg atgcctttga 1080
gggtggccgc atccatctgg tcagaaaaga caatcttttt gttgtcaagc ttggtggcaa 1140
acgacccgta gagggcgttg gacagcaact tggcgatgga gcgcagggtt tggtttttgt 1200
cgcgatcggc gcgctccttg gccgcgatgt ttagctgcac gtattcgcgc gcaacgcacc 1260
gccattcggg aaagacggtg gtgcgctcgt cgggcaccag gtgcacgcgc caaccgcggt 1320
tgtgcagggt gacaaggtca acgctggtgg ctacctctcc gcgtaggcgc tcgttggtcc 1380
agcagaggcg gccgcccttg cgcgagcaga atggcggtag ggggtctagc tgcgtctcgt 1440
ccggggggtc tgcgtccacg gtaaagaccc cgggcagcag gcgcgcgtcg aagtagtcta 1500
tcttgcatcc ttgcaagtct agcgcctgct gccatgcgcg ggcggcaagc gcgcgctcgt 1560
atgggttgag tgggggaccc catggcatgg ggtgggtgag cgcggaggcg tacatgccgc 1620
aaatgtcgta aacgtagagg ggctctctga gtattccaag atatgtaggg tagcatcttc 1680
caccgcggat gctggcgcgc acgtaatcgt atagttcgtg cgagggagcg aggaggtcgg 1740
gaccgaggtt gctacgggcg ggctgctctg ctcggaagac tatctgcctg aagatggcat 1800
gtgagttgga tgatatggtt ggacgctgga agacgttgaa gctggcgtct gtgagaccta 1860
ccgcgtcacg cacgaaggag gcgtaggagt cgcgcagctt gttgaccagc tcggcggtga 1920
cctgcacgtc tagggcgcag tagtccaggg tttccttgat gatgtcatac ttatcctgtc 1980
ccttttttttt ccacagctcg cggttgagga caaactcttc gcggtctttc cagtactctt 2040
ggatcggaaa cccgtcggcc tccgaacgag atccgtactc cgcccgccgag ggacctgagc 2100
gagtccgcat cgaccggatc ggaaaacctc tcgagaaagg cgtctaacca gtcacagtcg 2160

caagatccaa gatgaagcgc gcaagaccgt ctgaagatac cttcaacccc gtgtatccat 2220
atgacacgga aaccggtcct ccaactgtgc cttttcttac tcctcccttt gtatccccca 2280
atgggtttca agagagtccc cctggggtac tctctttgcg cctatccgaa cctctagtta 2340
cctccaatgg catgcttgcg ctcaaaatgg gcaacggcct ctctctggac gaggccggca 2400
accttacctc ccaaaatgta accactgtga gcccacctct caaaaaaacc aagtcaaaca 2460
taaacctgga aatatctgca cccctcacag ttacctcaga agccctaact gtggctgccg 2520
ccgcacctct aatggtcgcg ggcaacacac tcaccatgca atcacaggcc ccgctaaccg 2580
tgcacgactc caaacttagc attgccaccc aaggacccct cacagtgtca gaaggaaagc 2640
tagccctgca aacatcaggc cccctcacca ccaccgatag cagtaccctt actatcactg 2700
cctcaccccc tctaactact gccactggta gcttgggcat tgacttgaaa gagcccattt 2760
atacacaaaa tggaaaacta ggactaaagt acggggctcc tttgcatgta acagacgacc 2820
taaacacttt gaccgtagca actggtccag gtgtgactat taataatact tccttgcaaa 2880
ctaaagttac tggagccttg ggttttgatt cacaaggcaa tatgcaactt aatgtagcag 2940
gaggactaag gattgattct caaaacagac gccttatact tgatgttagt tatccgtttg 3000
atgctcaaaa ccaactaaat ctaagactag gacagggccc tctttttata aactcagccc 3060
acaacttgga tattaactac aacaaaggcc tttacttgtt tacagcttca aacaattcca 3120
aaaagcttga ggttaaccta agcactgcca aggggttgat gtttgacgct acagccatag 3180
ccattaatgc aggagatggg cttgaatttg gttcacctaa tgcaccaaac acaaatcccc 3240
tcaaaacaaa aattggccat ggcctagaat ttgattcaaa caaggctatg gttcctaaac 3300
taggaactgg ccttagtttt gacagcacag gtgccattac agtaggaaac aaaaataatg 3360
ataagctaac tttgtggacc ggtccaaaac cagaagccaa ctgcataatt gaatacggga 3420
aacaaaaccc agatagcaaa ctaactttaa tccttgtaaa aaatggagga attgttaatg 3480
gatatgtaac gctaatggga gcctcagact acgttaacac cttatttaaa aacaaaaatg 3540
tctccattaa tgtagaacta tactttgatg ccactggtca tatattacca gactcatctt 3600
ctcttaaaac agatctagaa ctaaaataca agcaaaccgc tgactttagt gcaagaggtt 3660
ttatgccaag tactacagcg tatccatttg tccttcctaa tgcgggaaca cataatgaaa 3720
attatatttt tggtcaatgc tactacaaag caagcgatgg tgcccttttt ccgttggaag 3780
ttactgttat gcttaataaa cgcctgccag atagtcgcac atcctatgtt atgacttttt 3840
tatggtcctt gaatgctggt ctagctccag aaactactca ggcaaccctc ataacctccc 3900
catttacctt ttcctatatt agagaagatg actgattttt aagaagcggc cgctcgagtc 3960
tagagggccc gtttaaaccc gctgatcagc ctcgactgtg ccttctagtt gccagccatc 4020
tgttgtttgc ccctcccccg tgccttcctt gaccctggaa ggtgccactc ccactgtcct 4080
ttcctaataa aatgaggaaa ttgcatcgca ttgtctgagt aggtgtcatt ctattctggg 4140
gggtggggtg gggcaggaca gcaaggggga ggattgggaa gacaatagca ggcatgctgg 4200
ggatgcggtg ggctctatgg cttctgaggc ggaaagaacc snccntagct ggggctctag 4260
ggggtatccc cacgcgccct gtagcggcgc attaagcgcg gcgggtgtgg tggttacgcg 4320
cagcgtgacc gctacacttg ccagcgccct agcgcccgct cctttcgctt tcttcccttc 4380
ctttctcgcc acgttcgccg gctttccccg tcaagctcta aatcggggca tccctttagg 4440
gttccgattt agtgctttac ggcacctcga ccccaaaaaa cttgattagg gtgatggttc 4500
acgtagtggg ccatcgccct gatagacggt ttttcgccct ttgacgttgg agtccacgtt 4560
ctttaatagt ggactcttgt tccaaactgg aacaacactc aaccctatct cggtctattc 4620
ttttgattta taagggattt tggggatttc ggcctattgg ttaaaaaatg agctgattta 4680
acaaaaattt aacgcgaatt aattctgtgg aatgtgtgtc agttagggtg tggaaagtcc 4740
ccaggctccc caggcaggca gaagtatgca aagcatgcat ctcaattagt cagcaaccag 4800
gtgtggaaag tccccaggct ccccagcagg cagaagtatg caaagcatgc atctcaatta 4860
gtcagcaacc atagtcccgc ccctaactcc gcccatcccg cccctaactc cgcccagttc 4920
cgcccattct ccgccccatg gctgactaat tttttttatt tatgcagagg ccgaggccgc 4980
ctctgcctct gagctattcc agaagtagtg aggaggcttt tttggaggcc taggcttttg 5040
caaaaagctc ccgggagctt gtatatccat tttcggatct gatcagcacg tgttgacaat 5100
taatcatcgg catagtatat cggcatagta taatacgaca aggtgaggaa ctaaaccatg 5160
gccaagttga ccagtgccgt tccggtgctc accgcgcgcg acgtcgccgg agcggtcgag 5220
ttctggaccg accggctcgg gttctcccgg gacttcgtgg aggacgactt cgccggtgtg 5280
gtccgggacg acgtgaccct gttcatcagc gcggtccagg accaggtggt gccggacaac 5340
accctggcct gggtgtgggt gcgcggcctg gacgagctgt acgccgagtg gtcggaggtc 5400
gtgtccacga acttccggga cgcctccggg ccggccatga ccgagatcgg cgagcagccg 5460
tgggggcggg agttcgccct gcgcgacccg gccggcaact gcgtgcactt cgtggccgag 5520
gagcaggact gacacgtgct acgagatttc gattccaccg ccgccttcta tgaaaggttg 5580
ggcttcggaa tcgttttccg ggacgccggc tggatgatcc tccagcgcgg ggatctcatg 5640
ctggagttct tcgcccaccc caacttgttt attgcagctt ataatggtta caaataaagc 5700
aatagcatca caaatttcac aaataaagca tttttttcac tgcattctag ttgtggtttg 5760
tccaaactca tcaatgtatc ttatcatgtc tgtataccgt cgacctctag ctagagcttg 5820
gcgtaatcat ggtcatagct gtttcctgtg tgaaattgt atccgctcac aattccacac 5880

aacatacgag ccggaagcat aaagtgtaaa gcctggggtg cctaatgagt gagctaactc 5940
acattaattg cgttgcgctc actgcccgct ttccagtcgg gaaacctgtc gtgccagctg 6000
cattaatgaa tcggccaacg cgcggggaga ggcggtttgc gtattgggcg ctcttccgct 6060
tcctcgctca ctgactcgct gcgctcggtc gttcggctgc ggcgagcggt atcagctcac 6120
tcaaaggcgg taatacggtt atccacagaa tcaggggata acgcaggaaa gaacatgtga 6180
gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg cgttgctggc gtttttccat 6240
aggctccgcc cccctgacga gcatcacaaa aatcgacgct caagtcagag gtggcgaaac 6300
ccgacaggac tataaagata ccaggcgttt ccccctggaa gctccctcgt gcgctctcct 6360
gttccgaccc tgccgcttac cggatacctg tccgcctttc tcccttcggg aagcgtggcg 6420
ctttctcaat gctcacgctg taggtatctc agttcggtgt aggtcgttcg ctccaagctg 6480
ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg ccttatccgg taactatcgt 6540
cttgagtcca acccggtaag acacgactta tcgccactgg cagcagccac tggtaacagg 6600
attagcagag cgaggtatgt aggcggtgct acagagttct tgaagtggtg gcctaactac 6660
ggctacacta gaaggacagt atttggtatc tgcgctctgc tgaagccagt taccttcgga 6720
aaaagagttg gtagctcttg atccggcaaa caaaccaccg ctggtagcgg tggttttttt 6780
gtttgcaagc agcagattac gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt 6840
tctacggggt ctgacgctca gtggaacgaa aactcacgtt aagggatttt ggtcatgaga 6900
ttatcaaaaa ggatcttcac ctagatcctt ttaaattaaa aatgaagttt taaatcaatc 6960
taaagtatat atgagtaaac ttggtctgac agttaccaat gcttaatcag tgaggcacct 7020
atctcagcga tctgtctatt tcgttcatcc atagttgcct gactccccgt cgtgtagata 7080
actacgatac gggagggctt accatctggc cccagtgctg caatgatacc gcgagaccca 7140
cgctcaccgg ctccagattt atcagcaata aaccagccag ccggaagggc cgagcgcaga 7200
agtggtcctg caactttatc cgcctccatc cagtctatta attgttgccg ggaagctaga 7260
gtaagtagtt cgccagttaa tagtttgcgc aacgttgttg ccattgctac aggcatcgtg 7320
gtgtcacgct cgtcgtttgg tatggcttca ttcagctccg gttcccaacg atcaaggcga 7380
gttacatgat cccccatgtt gtgcaaaaaa gcggttagct ccttcggtcc tccgatcgtt 7440
gtcagaagta agttggccgc agtgttatca ctcatggtta tggcagcact gcataattct 7500
cttactgtca tgccatccgt aagatgcttt tctgtgactg gtgagtactc aaccaagtca 7560
ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc cggcgtcaat acgggataat 7620
accgcgccac atagcagaac tttaaaagtg ctcatcattg gaaaacgttc ttcggggcga 7680
aaactctcaa ggatcttacc gctgttgaga tccagttcga tgtaacccac tcgtgcaccc 7740
aactgatctt cagcatcttt tactttcacc agcgtttctg ggtgagcaaa aacaggaagg 7800
caaaatgccg caaaaaaggg aataagggcg acacggaaat gttgaatact catactcttc 7860
ctttttcaat attattgaag catttatcag ggttattgtc tcatgagcgg atacatattt 7920
gaatgtattt agaaaaataa acaaataggg gttccgcgca catttccccg aaaagtgcca 7980
cctgacgtc 7989

<210> 34
<211> 7607
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid GRE5-E1-SV40-Hygro

<400> 34
tctagaagat ccgctgtaca ggatgttcta gctactttat tagatccgct gtacaggatg 60
ttctagctac tttattagat ccgctgtaca ggatgttcta gctactttat tagatccgct 120
gtacaggatg ttctagctac tttattagat ccgtgtacag gatgttctag ctactttatt 180
agatcgatct cctggccgtt cggggtcaaa aaccaggttt ggctataaaa gggggtgggg 240
gcgcgttcgt cctcactctc ttccgcatcg ctgtctgcga gggccaggat cgatcctgag 300
aacttcaggg tgagtttggg gacccttgat tgttctttct ttttcgctat tgtaaaattc 360
atgttatatg gagggggcaa agttttcagg gtgttgttta gaatgggaag atgtcccttg 420
tatcaccatg gaccctcatg ataattttgt ttctttcact ttctactctg ttgacaacca 480
ttgtctcctc ttattttctt ttcattttct gtaacttttt cgttaaactt tagcttgcat 540
ttgtaacgaa tttttaaatt cacttttgtt tatttgtcag attgtaagta ctttctctaa 600
tcactttttt ttcaaggcaa tcagggtata ttatattgta cttcagcaca gttttagaga 660
acaattgtta taattaaatg ataaggtaga atatttctgc atataaattc tggctggcgt 720
ggaaatattc ttattggtag aaacaactac atcctggtca tcatcctgcc tttctcttta 780
tggttacaat gatatacact gtttgagatg aggataaaat actctgagtc caaaccgggc 840
ccctctgcta accatgttca tgccttcttc tttttcctac agctcctggg caacgtgctg 900
gttattgtgc tgtctcatca ttttggcaaa gaattagatc taagcttctg cagctcgagg 960

```
actcggtcga ctgaaaatga gacatattat ctgccacgga ggtgttatta ccgaagaaat 1020
ggccgccagt cttttggacc agctgatcga agaggtactg gctgataatc ttccacctcc 1080
tagccatttt gaaccaccta cccttcacga actgtatgat ttagacgtga cggcccccga 1140
agatcccaac gaggaggcgg tttcgcagat ttttcccgac tctgtaatgt tggcggtgca 1200
ggaagggatt gacttactca cttttccgcc ggcgcccggt tctccggagc cgcctcacct 1260
ttcccggcag cccgagcagc cggagcagag agccttgggt ccggtttcta tgccaaacct 1320
tgtaccggag gtgatcgatc ttacctgcca cgaggctggc tttccaccca gtgacgacga 1380
ggatgaagag ggtgaggagt ttgtgttaga ttatgtggag caccccgggc acggttgcag 1440
gtcttgtcat tatcaccgga ggaatacggg ggacccagat attatgtgtt cgctttgcta 1500
tatgaggacc tgtggcatgt ttgtctacag taagtgaaaa ttatgggcag tgggtgatag 1560
agtggtgggt ttggtgtggt aatttttttt ttaattttta cagttttgtg gtttaaagaa 1620
ttttgtattg tgattttttt aaaaggtcct gtgtctgaac ctgagcctga gcccgagcca 1680
gaaccggagc ctgcaagacc tacccgccgt cctaaaatgg cgcctgctat cctgagacgc 1740
ccgacatcac ctgtgtctag agaatgcaat agtagtacgg atagctgtga ctccggtcct 1800
tctaacacac ctcctgagat acacccggtg gtcccgctgt gccccattaa accagttgcc 1860
gtgagagttg gtgggcgtcg ccaggctgtg gaatgtatcg aggacttgct taacgagcct 1920
gggcaacctt tggacttgag ctgtaaacgc cccaggccat aaggtgtaaa cctgtgattg 1980
cgtgtgtggt taacgccttt gtttgctgaa tgagttgatg taagtttaat aaagggtgag 2040
ataatgttta acttgcatgg cgtgttaaat ggggcggggc ttaaagggta tataatgcgc 2100
cgtgggctaa tcttggttac atctgacctc atggaggctt gggagtgttt ggaagatttt 2160
tctgctgtgc gtaacttgct ggaacagagc tctaacagta cctcttggtt ttggaggttt 2220
ctgtggggct catcccaggc aaagttagtc tgcagaatta aggaggatta caagtgggaa 2280
tttgaagagc ttttgaaatc ctgtggtgag ctgtttgatt ctttgaatct gggtcaccag 2340
gcgcttttcc aagagaaggt catcaagact ttggattttt ccacaccggg gcgcgctgcg 2400
gctgctgttg ctttttttgag ttttataaag gataaatgga gcgaagaaac ccatctgagc 2460
gggggggtacc tgctggattt tctggccatg catctgtgga gagcggttgt gagacacaag 2520
aatcgcctgc tactgttgtc ttccgtccgc ccggcgataa taccgacgga ggagcagcag 2580
cagcagcagg aggaagccag gcggcggcgg caggagcaga gcccatggaa cccgagagcc 2640
ggcctggacc ctcgggaatg aatgttgtac aggtggctga actgtatcca gaactgagac 2700
gcattttgac aattacagag gatgggcagg ggctaaaggg ggtaaagagg gagcgggggg 2760
cttgtgaggc tacagaggag gctaggaatc tagcttttag cttaatgacc agacaccgtc 2820
ctgagtgtat tacttttcaa cagatcaagg ataattgcgc taatgagctt gatctgctgg 2880
cgcagaagta ttccatagag cagctgacca cttactggct gcagccaggg gatgattttg 2940
aggaggctat tagggtatat gcaaaggtgg cacttaggcc agattgcaag tacaagatca 3000
gcaaacttgt aaatatcagg aattgttgct acatttctgg gaacggggcc gaggtggaga 3060
tagatacgga ggatagggtg gcctttagat gtagcatgat aaatatgtgg ccgggggtgc 3120
ttggcatgga cggggtggtt attatgaatg taaggtttac tggccccaat tttagcggta 3180
cggttttcct ggccaatacc aaccttatcc tacacggtgt aagcttctat gggtttaaca 3240
atacctgtgt ggaagcctgg accgatgtaa gggttcgggg ctgtgccttt tactgctgct 3300
ggaagggggt ggtgtgtcgc cccaaaagca gggcttcaat taagaaatgc ctctttgaaa 3360
ggtgtacctt gggtatcctg tctgagggta actccagggt gcgccacaat gtggcctccg 3420
actgtggttg cttcatgcta gtgaaaagcg tggctgtgat taagcataac atggtatgtg 3480
gcaactgcga ggacagggcc tctcagatgc tgacctgctc ggacggcaac tgtcacctgc 3540
tgaagaccat tcacgtagcc agccactctc gcaaggcctg gccagtgttt gagcataaca 3600
tactgacccg ctgttccttg catttgggta acaggagggg ggtgttccta ccttaccaat 3660
gcaatttgag tcacactaag atattgcttg agcccgagag catgtccaag gtgaacctga 3720
acggggtgtt tgacatgacc atgaagatct ggaaggtgct gaggtacgat gagacccgca 3780
ccaggtgcag accctgcgag tgtggcggta aacatattag gaaccagcct gtgatgctgg 3840
atgtgaccga ggagctgagg cccgatcact tggtgctggc ctgcacccgc gctgagtttg 3900
gctctagcga tgaagataca gattgaggta ctgaaatgtg tgggcgtggc ttaagggtgg 3960
gaaagaatat ataaggtggg ggtcttatgt agttttgtat ctgttttgca gcagccgccg 4020
ccgccatgag caccaactcg tttgatggaa gcattgtgag ctcatatttg acaacgcgca 4080
tgcccccatg ggccggggtg cgtcagaatg tgatgggctc cagcattgat ggtcgccccg 4140
tcctgcccgc aaactctact accttgacct acgagaccgt gtctggaacg ccgttggaga 4200
ctgcagcctc cgccgccgct tcagccgctg cagccaccgc ccgcgggatt gtgactgact 4260
ttgctttcct gagcccgctt gcaagcagtg cagcttcccg ttcatccgcc cgcgatgaca 4320
agttgacggc tcttttggca caattggatt ctttgacccg ggaacttaat gtcgtttctc 4380
agcagctgtt ggatctgcgc cagcaggttt ctgccctgaa ggcttcctcc cctcccaatg 4440
cggtttaaaa cataaataaa aaaccagact ctgtttggat ttggatcaag caagtgtctt 4500
gctgtctcag ctgactgctt aagtcgcaag ccgaattgga tccaattcgg atcgatctta 4560
ttaaagcaga acttgtttat tgcagcttat aatggttaca aataaagcaa tagcatcaca 4620
aatttcacaa ataaagcatt tttttcactg cattctagtt gtggtttgtc caaactcatc 4680
```

aatgtatctt atcatgtctg gtcgactcta gactcttccg cttcctcgct cactgactcg 4740
ctgcgctcgg tcgttcggct gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg 4800
ttatccacag aatcagggga taacgcagga aagaacatgt gagcaaaagg ccagcaaaag 4860
gccaggaacc gtaaaaaggc cgcgttgctg gcgtttttcc ataggctccg cccccctgac 4920
gagcatcaca aaaatcgacg ctcaagtcag aggtggcgaa acccgacagg actataaaga 4980
taccaggcgt ttccccctgg aagctccctc gtgcgctctc ctgttccgac cctgccgctt 5040
accggatacc tgtccgcctt tctcccttcg ggaagcgtgg cgctttctca tagctcacgc 5100
tgtaggtatc tcagttcggt gtaggtcgtt cgctccaagc tgggctgtgt gcacgaaccc 5160
cccgttcagc ccgaccgctg cgccttatcc ggtaactatc gtcttgagtc caacccggta 5220
agacacgact tatcgccact ggcagcagcc actggtaaca ggattagcag agcgaggtat 5280
gtaggcggtg ctacagagtt cttgaagtgg tggcctaact acggctacac tagaaggaca 5340
gtatttggta tctgcgctct gctgaagcca gttaccttcg gaaaaagagt tggtagctct 5400
tgatccggca aacaaaccac cgctggtagc ggtggttttt ttgtttgcaa gcagcagatt 5460
acgcgcagaa aaaaaggatc tcaagaagat cctttgatct tttctacggg gtctgacgct 5520
cagtggaacg aaaactcacg ttaagggatt ttggtcatga gattatcaaa aaggatcttc 5580
acctagatcc ttttaaatta aaaatgaagt tttaaatcaa tctaaagtat atatgagtaa 5640
acttggtctg acagttacca atgcttaatc agtgaggcac ctatctcagc gatctgtcta 5700
tttcgttcat ccatagttgc ctgactcccc gtcgtgtaga taactacgat acgggagggc 5760
ttaccatctg gccccagtgc tgcaatgata ccgcgagacc cacgctcacc ggctccagat 5820
ttatcagcaa taaaccagcc agccggaagg gccgagcgca gaagtggtcc tgcaacttta 5880
tccgcctcca tccagtctat taattgttgc cgggaagcta gagtaagtag ttcgccagtt 5940
aatagtttgc gcaacgttgt tgccattgct acaggcatcg tggtgtcacg ctcgtcgttt 6000
ggtatggctt cattcagctc cggttcccaa cgatcaaggc gagttacatg atcccccatg 6060
ttgtgcaaaa aagcggttag ctccttcggt cctccgatcg ttgtcagaag taagttggcc 6120
gcagtgttat cactcatggt tatggcagca ctgcataatt ctcttactgt catgccatcc 6180
gtaagatgct tttctgtgac tggtgagtac tcaaccaagt cattctgaga atagtgtatg 6240
cggcgaccga gttgctcttg cccggcgtca atacgggata ataccgcgcc acatagcaga 6300
actttaaaag tgctcatcat tggaaaacgt tcttcggggc gaaaactctc aaggatctta 6360
ccgctgttga gatccagttc gatgtaaccc actcgtgcac ccaactgatc ttcagcatct 6420
tttactttca ccagcgtttc tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag 6480
ggaataaggg cgacacggaa atgttgaata ctcatactct tcctttttca atattattga 6540
agcatttatc agggttattg tctcatgagc ggatacatat ttgaatgtat ttagaaaaat 6600
aaacaaatag gggttccgcg cacatttccc cgaaaagtgc cacctgacgt ctaagaaacc 6660
attattatca tgacattaac ctataaaaat aggcgtatca cgaggccct ttcgtctcgc 6720
gcgtttcggt gatgacggtg aaaacctctg acacatgcag ctcccggaga cggtcacagc 6780
ttgtctgtaa gcggatgccg ggagcagaca agcccgtcag ggcgcgtcag cgggtgttgg 6840
cgggtgtcgg ggctggctta actatgcggc atcagagcag attgtactga gagtgcacca 6900
tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca ggaaattgta 6960
agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc attttttaac 7020
caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga gatagggttg 7080
agtgttgttc cagtttggaa caagagtcca ctattaaaga acgtggactc caacgtcaaa 7140
gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc ctaatcaagt 7200
tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaagggag cccccgattt 7260
agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa agcgaaagga 7320
gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac cacacccgcc 7380
gcgcttaatg cgccgctaca gggcgcgtcc cattcgccat tcaggctgcg caactgttgg 7440
gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg gggatgtgct 7500
gcaaggcgat taagttgggt aacgccaggg ttttcccagt cacgacgttg taaaacgacg 7560
gccagtgaat tgtaatacga ctcactatag ggcgaattaa ttcgggg 7607

<210> 35
<211> 11600
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid MMTV-E2a-SV40-Neo

<400> 35
gaattccgca ttgcagagat attgtattta agtgcctagc tcgatacaat aaacgccatt 60
tgaccattca ccacattggt gtgcacctcc aagcttgggc agaaatggtt gaactcccga 120

gagtgtccta cacctagggg agaagcagcc aaggggttgt ttcccaccaa ggacgacccg 180
tctgcgcaca aacggatgag cccatcagac aaagacatat tcattctctg ctgcaaactt 240
ggcatagctc tgctttgcct ggggctattg ggggaagttg cggttcgtgc tcgcagggct 300
ctcacccttg actcttttaa tagctcttct gtgcaagatt acaatctaaa caattcggag 360
aactcgacct tcctcctgag gcaaggacca cagccaactt cctcttacaa gccgcatcga 420
ttttgtcctt cagaaataga aataagaatg cttgctaaaa attatatttt taccaataag 480
accaatccaa taggtagatt attagttact atgttaagaa atgaatcatt atcttttagt 540
actattttta ctcaaattca gaagttagaa atgggaatag aaaatagaaa gagacgctca 600
acctcaattg aagaacaggt gcaaggacta ttgaccacag gcctagaagt aaaaaaggga 660
aaaaagagtg tttttgtcaa aataggagac aggtggtggc aaccagggac ttatagggga 720
ccttacatct acagaccaac agatgccccc ttaccatata caggaagata tgacttaaat 780
tgggataggt gggttacagt caatggctat aaagtgttat atagatccct ccctttttcgt 840
gaaagactcg ccagagctag acctccttgg tgtatgttgt ctcaagaaga aaaagacgac 900
atgaaacaac aggtacatga ttatatttat ctaggaacag gaatgcactt ttggggaaag 960
attttccata ccaaggaggg gacagtggct ggactaatag aacattattc tgcaaaaact 1020
catggcatga gttattatga atagccttta ttggcccaac cttgcggttc ccagggctta 1080
agtaagtttt tggttacaaa ctgttcttaa aacgaggatg tgagacaagt ggtttcctga 1140
cttggtttgg tatcaaaggt tctgatctga gctctgagtg ttctattttc ctatgttctt 1200
ttggaattta tccaaatctt atgtaaatgc ttatgtaaac caagatataa aagagtgctg 1260
atttttgag taaacttgca acagtcctaa cattcacctc ttgtgtgttt gtgtctgttc 1320
gccatcccgt ctccgctcgt cacttatcct tcactttcca gagggtcccc ccgcagaccc 1380
cggcgaccct caggtcggcc gactgcggca gctggcgccc gaacagggac cctcggataa 1440
gtgacccttg tctctatttc tactatttgg tgtttgtctt gtattgtctc tttcttgtct 1500
ggctatcatc acaagagcgg aacggactca ccatagggac caagctagcg cttctcgtcg 1560
cgtccaagac cctcaaagat ttttggcact tcgttgagcg aggcgatatc aggtatgaca 1620
gcgccctgcc gcaaggccag ctgcttgtcc gctcggctgc ggttggcacg gcaggatagg 1680
ggtatcttgc agttttggaa aaagatgtga taggtggcaa gcacctctgg cacggcaaat 1740
acggggtaga agttgaggcg cgggttgggc tcgcatgtgc cgttttcttg gcgtttgggg 1800
ggtacgcgcg gtgagaatag gtggcgttcg taggcaaggc tgacatccgc tatggcgagg 1860
ggcacatcgc tgcgctcttg caacgcgtcg cagataatgg cgcactggcg ctgcagatgc 1920
ttcaacagca cgtcgtctcc cacatctagg tagtcgccat gcctttcgtc cccccgcccg 1980
acttgttcct cgtttgcctc tgcgttgtcc tggtcttgct ttttatcctc tgttggtact 2040
gagcggtcct cgtcgtcttc gcttacaaaa cctgggtcct gctcgataat cacttcctcc 2100
tcctcaagcg ggggtgcctc gacggggaag gtggtaggcg cgttggcggc atcggtggag 2160
gcggtggtgg cgaactcaga gggggcggtt aggctgtcct tcttctcgac tgactccatg 2220
atctttttct gcctatagga gaaggaaatg gccagtcggg aagaggagca gcgcgaaacc 2280
accccсgagc gcggacgcgg tgcggcgcga cgtcccccaa ccatggagga cgtgtcgtcc 2340
ccgtccccgt cgccgccgcc tccccgggcg cccccaaaaa agcggatgag gcggcgtatc 2400
gagtccgagg acgaggaaga ctcatcacaa gacgcgctgg tgccgcgcac acccagcccg 2460
cggccatcga cctcggcggc ggatttggcc attgcgccca agaagaaaaa gaagcgccct 2520
tctcccaagc ccgagcgccc gccatcacca gaggtaatcg tggacagcga ggaagaaaga 2580
gaagatgtgg cgctacaaat ggtgggtttc agcaacccac cggtgctaat caagcatggc 2640
aaaggaggta agcgcacagt gcggcggctg aatgaagacg acccagtggc gcgtggtatg 2700
cggacgcaag aggaagagga agagcccagc gaagcggaaa gtgaaattac ggtgatgaac 2760
ccgctgagtg tgccgatcgt gtctgcgtgg gagaagggca tggaggctgc gcgcgcgctg 2820
atggacaagt accacgtgga taacgatcta aaggcgaact tcaaactact gcctgaccaa 2880
gtggaagctc tggcggccgt atgcaagacc tggctgaacg aggagcaccg cgggttgcag 2940
ctgaccttca ccagcaacaa gacctttgtg acgatgatgg ggcgattcct gcaggcgtac 3000
ctgcagtcgt ttgcagaggt gacctacaag catcacgagc ccacgggctg cgcgttgtgg 3060
ctgcaccgct gcgctgagat cgaaggcgag cttaagtgtc tacacggaag cattatgata 3120
aataaggagc acgtgattga aatggatgtg acgagcgaaa acgggcagcg cgcgctgaag 3180
gagcagtcta gcaaggccaa gatcgtgaag aaccggtggg gccgaaatgt ggtgcagatc 3240
tccaacaccg acgcaaggtg ctgcgtgcac gacgcggcct gtccggccaa tcagttttcc 3300
ggcaagtctt gcggcatgtt cttctctgaa ggcgcaaagg ctcaggtggc ttttaagcag 3360
atcaaggctt ttatgcaggc gctgtatcct aacgcccaga ccgggcacgg tcaccttttg 3420
atgccactac ggtgcgagtg caactcaaag cctgggcacg cgcccttttt gggaaggcag 3480
ctaccaaagt tgactccgtt cgccctgagc aacgcggagg acctggacgc ggatctgatc 3540
tccgacaaga gcgtgctggc cagcgtgcac caccсggcgc tgatagtgtt ccagtgctgc 3600
aaccctgtgt atcgcaactc gcgcgcgcag ggcggaggcc ccaactgcga cttcaagata 3660
tcggcgcccg acctgctaaa cgcgttggtg atggtgcgca gcctgtggag tgaaaacttc 3720
accgagctgc cgcggatggt tgtgcctgag tttaagtgga gcactaaaca ccagtatcgc 3780
aacgtgtccc tgccagtggc gcatagcgat gcgcggcaga acccctttga tttttaaacg 3840

gcgcagacgg caagggtggg ggtaaataat cacccgagag tgtacaaata aaagcatttg 3900
cctttattga aagtgtctct agtacattat ttttacatgt ttttcaagtg acaaaaagaa 3960
gtggcgctcc taatctgcgc actgtggctg cggaagtagg gcgagtggcg ctccaggaag 4020
ctgtagagct gttcctggtt gcgacgcagg gtgggctgta cctggggact gttgagcatg 4080
gagttgggta ccccggtaat aaggttcatg gtggggttgt gatccatggg agtttggggc 4140
cagttggcaa aggcgtggag aaacatgcag cagaatagtc cacaggcggc cgagttgggc 4200
ccctgtacgc tttgggtgga cttttccagc gttatacagc ggtcgggga agaagcaatg 4260
gcgctacggc gcaggagtga ctcgtactca aactggtaaa cctgcttgag tcgctggtca 4320
gaaaagccaa agggctcaaa gaggtagcat gtttttgagt gcgggttcca ggcaaaggcc 4380
atccagtgta cgcccccagt ctcgcgaccg gccgtattga ctatggcgca ggcgagcttg 4440
tgtggagaaa caaagcctgg aaagcgcttg tcataggtgc ccaaaaaata tggcccacaa 4500
ccaagatctt tgacaatggc tttcagttcc tgctcactgg agcccatggc ggcagctgtt 4560
gttgatgttg cttgcttctt tatgttgtgg cgttgccggc cgagaagggc gtgcgcaggt 4620
acacggtttc gatgacgccg cggtgcggcc ggtgcacacg gaccacgtca aagacttcaa 4680
acaaaacata aagaagggtg ggctcgtcca tgggatccat atatagggcc cgggttataa 4740
ttacctcagg tcgacctcga gggatctttg tgaaggaacc ttacttctgt ggtgtgacat 4800
aattggacaa actacctaca gagatttaaa gctctaaggt aaatataaaa tttttaagtg 4860
tataatgtgt taaactactg attctaattg tttgtgtatt ttagattcca acctatggaa 4920
ctgatgaatg ggagcagtgg tggaatgcct ttaatgagga aaacctgttt tgctcagaag 4980
aaatgccatc tagtgatgat gaggctactg ctgactctca acattctact cctccaaaaa 5040
agaagagaaa ggtagaagac cccaaggact ttccttcaga attgctaagt tttttgagtc 5100
atgctgtgtt tagtaataga actcttgctt gctttgctat ttacaccaca aaggaaaaag 5160
ctgcactgct atacaagaaa attatggaaa aatattctgt aacctttata agtaggcata 5220
acagttataa tcataacata ctgttttttc ttactccaca caggcataga gtgtctgcta 5280
ttaataacta tgctcaaaaa ttgtgtacct ttagcttttt aatttgtaaa ggggttaata 5340
aggaatattt gatgtatagt gccttgacta gagatcataa tcagccatac cacatttgta 5400
gaggttttac ttgctttaaa aaacctccca cacctccccc tgaacctgaa acataaaatg 5460
aatgcaattg ttgttgttaa cttgtttatt gcagcttata atggttacaa ataaagcaat 5520
agcatcacaa atttcacaaa taaagcattt ttttcactgc attctagttg tggtttgtcc 5580
aaactcatca atgtatctta tcatgtctgg atccggctgt ggaatgtgtg tcagttaggg 5640
tgtggaaagt ccccaggctc cccagcaggc agaagtatgc aaagcatgca tctcaattag 5700
tcagcaacca ggtgtggaaa gtccccaggc tccccagcag gcagaagtat gcaaagcatg 5760
catctcaatt agtcagcaac catagtcccg cccctaactc cgcccatccc gcccctaact 5820
ccgcccagtt ccgcccattc tccgccccat ggctgactaa ttttttttat ttatgcagag 5880
gccgaggccg cctcggcctc tgagctattc cagaagtagt gaggaggctt ttttggaggc 5940
ctaggctttt gcaaaaagct tcacgctgcc gcaagcactc agggcgcaag ggctgctaaa 6000
ggaagcggaa cacgtagaaa gccagtccgc agaaacggtg ctgaccccgg atgaatgtca 6060
gctactgggc tatctggaca agggaaaacg caagcgcaaa gagaaagcag gtagcttgca 6120
gtgggcttac atggcgatag ctagactggg cggttttatg gacagcaagc gaaccggaat 6180
tgccagctgg ggcgccctct ggtaaggttg ggaagccctg caaagtaaac tggatggctt 6240
tcttgccgcc aaggatctga tggcgcaggg gatcaagatc tgatcaagag acaggatgag 6300
gatcgtttcg catgattgaa caagatggat tgcacgcagg ttctccggcc gcttgggtgg 6360
agaggctatt cggctatgac tgggcacaac agacaatcgg ctgctctgat gccgccgtgt 6420
tccggctgtc agcgcagggg cgcccggttc tttttgtcaa gaccgacctg tccggtgccc 6480
tgaatgaact gcaggacgag gcagcgcggc tatcgtggct ggccacgacg ggcgttcctt 6540
gcgcagctgt gctcgacgtt gtcactgaag cgggaaggga ctggctgcta ttgggcgaag 6600
tgccggggca ggatctcctg tcatctcacc ttgctcctgc cgagaaagta tccatcatgg 6660
ctgatgcaat gcggcggctg catacgcttg atccggctac ctgcccattc gaccaccaag 6720
cgaaacatcg catcgagcga gcacgtactc ggatggaagc cggtcttgtc gatcaggatg 6780
atctggacga agagcatcag gggctcgcgc cagccgaact gttcgccagg ctcaaggcgc 6840
gcatgcccga cggcgaggat ctcgtcgtga cccatggcga tgcctgcttg ccgaatatca 6900
tggtggaaaa tggccgcttt tctggattca tcgactgtgg ccggctgggt gtggcggacc 6960
gctatcagga catagcgttg gctacccgtg atattgctga agagcttggc ggcgaatggg 7020
ctgaccgctt cctcgtgctt tacggtatcg ccgctcccga ttcgcagcgc atcgccttct 7080
atcgccttct tgacgagttc ttctgagcgg gactctgggg ttcgaaatga ccgaccaagc 7140
gacgcccaac ctgccatcac gagatttcga ttccaccgcc gccttctatg aaaggttggg 7200
cttcggaatc gttttccggg acgccggctg gatgatcctc cagcgcgggg atctcatgct 7260
ggagttcttc gcccaccccg ggctcgatcc cctcgcgagt tggttcagct gctgcctgag 7320
gctggacgac ctcgcggagt tctaccggca gtgcaaatcc gtcggcatcc aggaaaccag 7380
cagcggctat ccgcgcatcc atgcccccga actgcaggag tggggaggca cgatggccgc 7440
tttggtcccg gatctttgtg aaggaacctt acttctgtgg tgtgacataa ttggacaaac 7500
tacctacaga gatttaaagc tctaaggtaa atataaaatt tttaagtgta taatgtgtta 7560

```
aactactgat tctaattgtt tgtgtatttt agattccaac ctatggaact gatgaatggg 7620
agcagtggtg gaatgccttt aatgaggaaa acctgttttg ctcagaagaa atgccatcta 7680
gtgatgatga ggctactgct gactctcaac attctactcc tccaaaaaag aagagaaagg 7740
tagaagaccc caaggacttt ccttcagaat tgctaagttt tttgagtcat gctgtgttta 7800
gtaatagaac tcttgcttgc tttgctattt acaccacaaa ggaaaaagct gcactgctat 7860
acaagaaaat tatggaaaaa tattctgtaa cctttataag taggcataac agttataatc 7920
ataacatact gtttttttctt actccacaca ggcatagagt gtctgctatt aataactatg 7980
ctcaaaaatt gtgtaccttt agctttttaa tttgtaaagg ggttaataag gaatatttga 8040
tgtatagtgc cttgactaga gatcataatc agccatacca catttgtaga ggttttactt 8100
gctttaaaaa acctcccaca cctccccctg aacctgaaac ataaaatgaa tgcaattgtt 8160
gttgttaact tgtttattgc agcttataat ggttacaaat aaagcaatag catcacaaat 8220
ttcacaaata aagcattttt ttcactgcat tctagttgtg gtttgtccaa actcatcaat 8280
gtatcttatc atgtctggat ccccaggaag ctcctctgtg tcctcataaa ccctaacctc 8340
ctctacttga gaggacattc caatcatagg ctgcccatcc accctctgtg tcctcctgtt 8400
aattaggtca cttaacaaaa aggaaattgg gtaggggttt ttcacagacc gctttctaag 8460
ggtaatttta aaatatctgg gaagtccctt ccactgctgt gttccagaag tgttggtaaa 8520
cagcccacaa atgtcaacag cagaaacata caagctgtca gctttgcaca agggcccaac 8580
accctgctca tcaagaagca ctgtggttgc tgtgttagta atgtgcaaaa caggaggcac 8640
attttcccca cctgtgtagg ttccaaaata tctagtgttt tcatttttac ttggatcagg 8700
aacccagcac tccactggat aagcattatc cttatccaaa acagccttgt ggtcagtgtt 8760
catctgctga ctgtcaactg tagcattttt tggggttaca gtttgagcag gatatttggt 8820
cctgtagttt gctaacacac cctgcagctc caaaggttcc ccaccaacag caaaaaaatg 8880
aaaatttgac ccttgaatgg gttttccagc accattttca tgagtttttt gtgtccctga 8940
atgcaagttt aacatagcag ttaccccaat aacctcagtt ttaacagtaa cagcttccca 9000
catcaaaata tttccacagg ttaagtcctc atttaaatta ggcaaaggaa ttcttgaaga 9060
cgaaagggcc tcgtgatacg cctattttta taggttaatg tcatgataat aatggtttct 9120
tagacgtcag gtggcacttt tcggggaaat gtgcgcggaa cccctatttg tttatttttc 9180
taaatacatt caaatatgta tccgctcatg agacaataac cctgataaat gcttcaataa 9240
tattgaaaaa ggaagagtat gagtattcaa catttccgtg tcgcccttat tccctttttt 9300
gcggcatttt gccttcctgt ttttgctcac ccagaaacgc tggtgaaagt aaaagatgct 9360
gaagatcagt tgggtgcacg agtgggttac atcgaactgg atctcaacag cggtaagatc 9420
cttgagagtt ttcgccccga agaacgtttt ccaatgatga gcacttttaa agttctgcta 9480
tgtggcgcgg tattatcccg tgttgacgcc gggcaagagc aactcggtcg ccgcatacac 9540
tattctcaga atgacttggt tgagtactca ccagtcacag aaaagcatct tacggatggc 9600
atgacagtaa gagaattatg cagtgctgcc ataaccatga gtgataacac tgcggccaac 9660
ttacttctga caacgatcgg aggaccgaag gagctaaccg cttttttgca caacatgggg 9720
gatcatgtaa ctcgccttga tcgttgggaa ccggagctga atgaagccat accaaacgac 9780
gagcgtgaca ccacgatgcc tgcagcaatg gcaacaacgt tgcgcaaact attaactggc 9840
gaactactta ctctagcttc ccggcaacaa ttaatagact ggatggaggc ggataaagtt 9900
gcaggaccac ttctgcgctc ggcccttccg gctggctggt ttattgctga taaatctgga 9960
gccggtgagc gtgggtctcg cggtatcatt gcagcactgg ggccagatgg taagccctcc 10020
cgtatcgtag ttatctacac gacggggagt caggcaacta tggatgaacg aaatagacag 10080
atcgctgaga taggtgcctc actgattaag cattggtaac tgtcagacca agtttactca 10140
tatatacttt agattgattt aaaacttcat ttttaattta aaaggatcta ggtgaagatc 10200
ctttttgata atctcatgac caaaatccct taacgtgagt tttcgttcca ctgagcgtca 10260
gaccccgtag aaaagatcaa aggatcttct tgagatcctt tttttctgcg cgtaatctgc 10320
tgcttgcaaa caaaaaaacc accgctacca gcggtggttt gtttgccgga tcaagagcta 10380
ccaactcttt ttccgaaggt aactggcttc agcagagcgc agataccaaa tactgtcctt 10440
ctagtgtagc cgtagttagg ccaccacttc aagaactctg tagcaccgcc tacatacctc 10500
gctctgctaa tcctgttacc agtggctgct gccagtggcg ataagtcgtg tcttaccggg 10560
ttggactcaa gacgatagtt accggataag gcgcagcggt cgggctgaac ggggggttcg 10620
tgcacacagc ccagcttgga gcgaacgacc tacaccgaac tgagatacct acagcgtgag 10680
ctatgagaaa gcgccacgct tcccgaaggg agaaaggcgg acaggtatcc ggtaagcggc 10740
agggtcggaa caggagagcg cacgagggag cttccagggg gaaacgcctg gtatctttat 10800
agtcctgtcg ggtttcgcca cctctgactt gagcgtcgat ttttgtgatg ctcgtcaggg 10860
gggcggagcc tatggaaaaa cgccagcaac gcggcctttt tacggttcct ggccttttgc 10920
tggccttttg ctcacatgtt ctttcctgcg ttatcccctg attctgtgga taaccgtatt 10980
accgcctttg agtgagctga taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca 11040
gtgagcgagg aagcggaaga gcgcctgatg cggtattttc tccttacgca tctgtgcggt 11100
atttcacacc gcatatggtg cactctcagt acaatctgct ctgatgccgc atagttaagc 11160
cagtatctgc tccctgcttg tgtgttggag gtcgctgagt agtgcgcgag caaaatttaa 11220
gctacaacaa ggcaaggctt gaccgacaat tgcatgaaga atctgcttag ggttaggcgt 11280
```

tttgcgctgc ttcgcgatgt acgggccaga tatacgcgta tctgagggga ctagggtgtg 11340
tttaggcgaa aagcggggct tcggttgtac gcggttagga gtcccctcag gatatagtag 11400
tttcgctttt gcatagggag ggggaaatgt agtcttatgc aatacacttg tagtcttgca 11460
acatggtaac gatgagttag caacatgcct tacaaggaga gaaaaagcac cgtgcatgcc 11520
gattggtgga agtaaggtgg tacgatcgtg ccttattagg aaggcaacag acgggtctga 11580
catggattgg acgaaccact 11600

<210> 36
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 36
gtcactcgag gactcggtcg actgaaaatg agacatatta tctgccacgg acc 53

<210> 37
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 37
cgagatcgat cacctccggt acaaggtttg gcatag 36

<210> 38
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 38
catgaagatc tggaaggtgc tgaggtacga tgagacc 37

<210> 39
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 39
gcgacttaag cagtcagctg agacagcaag acacttgctt gatccaaatc c 51

<210> 40
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 40
cacgaattcg tcagcgcttc tcgtcgcgtc caagaccc 38

<210> 41
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 41
caccccgggg aggcggcggc gacggggacg gg 32

<210> 42
<211> 7231
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: plasmid pDV80

<400> 42
ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg cgagcaaaat ttaagctaca 60
acaaggcaag gcttgaccga caattgcatg aagaatctgc ttagggttag gcgttttgcg 120
ctgcttcgcg atgtacgggc cagatatacg cgttgacatt gattattgac tagttattaa 180
tagtaatcaa ttacggggtc attagttcat agcccatata tggagttccg cgttacataa 240
cttacggtaa atggcccgcc tggctgaccg cccaacgacc cccgcccatt gacgtcaata 300
atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca atgggtggac 360
tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc aagtacgccc 420
cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta catgacctta 480
tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac catggtgatg 540
cggttttggc agtacatcaa tgggcgtgga tagcggtttg actcacgggg atttccaagt 600
ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg ggactttcca 660
aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt acggtgggag 720
gtctatataa gcagagctct ctggctaact agagaaccca ctgcttactg gcttatcgaa 780
attaatacga ctcactatag ggagacccaa gctggctagc gtttaaactt aagcttggta 840
ccgagctcgg atccactctc ttccgcatcg ctgtctgcga gggccagctg ttggggtgag 900
tactccctct gaaaagcggg catgacttct gcgctaagat tgtcagtttc caaaaacgag 960
gaggatttga tattcacctg gcccgcggtg atgcctttga gggtggccgc atccatctgg 1020
tcagaaaaga caatcttttt gttgtcaagc ttggtggcaa acgacccgta gagggcgttg 1080
gacagcaact tggcgatgga gcgcagggtt tggtttttgt cgcgatcggc gcgctccttg 1140
gccgcgatgt ttagctgcac gtattcgcgc gcaacgcacc gccattcggg aaagacggtg 1200
gtgcgctcgt cgggcaccag gtgcacgcgc caaccgcggt tgtgcagggt gacaaggtca 1260
acgctggtgg ctacctctcc gcgtaggcgc tcgttggtcc agcagaggcg gccgcccttg 1320
cgcgagcaga atggcggtag ggggtctagc tgcgtctcgt ccggggggtc tgcgtccacg 1380
gtaaagaccc cgggcagcag gcgcgcgtcg aagtagtcta tcttgcatcc ttgcaagtct 1440
agcgcctgct gccatgcgcg ggcggcaagc gcgcgctcgt atgggttgag tgggggaccc 1500
catggcatgg ggtgggtgag cgcggaggcg tacatgccgc aaatgtcgta aacgtagagg 1560
ggctctctga gtattccaag atatgtaggg tagcatcttc caccgcggat gctggcgcgc 1620
acgtaatcgt atagttcgtg cgagggagcg aggaggtcgg gaccgaggtt gctacgggcg 1680
ggctgctctg ctcggaagac tatctgcctg aagatggcat gtgagttgga tgatatggtt 1740
ggacgctgga agacgttgaa gctggcgtct gtgagaccta ccgcgtcacg cacgaaggag 1800
gcgtaggagt cgcgcagctt gttgaccagc tcggcggtga cctgcacgtc tagggcgcag 1860
tagtccaggg tttccttgat gatgtcatac ttatcctgtc cctttttttt ccacagctcg 1920
cggttgagga caaactcttc gcggtctttc cagtactctt ggatcggaaa cccgtcggcc 1980
tccgaacgag atccgtactc cgccgccgag ggacctgagc gagtccgcat cgaccggatc 2040
ggaaaacctc tcgagaaagg cgtctaacca gtcacagtcg caagatccaa gatgaagcgc 2100
gccccgcccca gcgaagatga cttcaacccc gtctacccct atggctacgc gcggaatcag 2160
aatatcccct tcctcactcc ccccttttgtc tcctccgatg gattcaaaaa cttccccccct 2220
ggggtactgt cactcaaact ggctgatcca atcaccatta ccaatgggga tgtatccctc 2280
aaggtgggag gtggtctcac tttgcaagat ggaagcctaa ctgtaaaccc taaggctcca 2340
ctgcaagtta atactgataa aaaacttgag cttgcatatg ataatccatt tgaaagtagt 2400
gctaataaac ttagtttaaa agtaggacat ggattaaaag tattagatga aaaaagtgct 2460

```
gcggggttaa aagatttaat tggcaaactt gtggttttaa caggaaaagg aataggcact 2520
gaaaatttag aaaatacaga tggtagcagc agaggaattg gtataaatgt aagagcaaga 2580
gaagggttga catttgacaa tgatggatac ttggtagcat ggaacccaaa gtatgacacg 2640
cgcacacttt ggacaacacc agacacatct ccaaactgca caattgctca agataaggac 2700
tctaaactca ctttggtact tacaaagtgt ggaagtcaaa tattagctaa tgtgtctttg 2760
attgtggtcg caggaaagta ccacatcata aataataaga caaatccaaa aataaaaagt 2820
tttactatta aactgctatt taataagaac ggagtgcttt tagacaactc aaatcttgga 2880
aaagcttatt ggaactttag aagtggaaat tccaatgttt cgacagctta tgaaaaagca 2940
attggtttta tgcctaattt ggtagcgtat ccaaaaccca gtaattctaa aaaatatgca 3000
agagacatag tttatggaac tatatatctt ggtggaaaac ctgatcagcc agcagtcatt 3060
aaaactacct ttaaccaaga aactggatgt gaatactcta tcacatttaa ctttagttgg 3120
tccaaaacct atgaaaatgt tgaatttgaa accacctctt ttaccttctc ctatattgcc 3180
caagaatgaa agagcggccg ctcgagtcta gagggcccgt ttaaacccgc tgatcagcct 3240
cgactgtgcc ttctagttgc cagccatctg ttgtttgccc ctcccccgtg ccttccttga 3300
ccctggaagg tgccactccc actgtccttt cctaataaaa tgaggaaatt gcatcgcatt 3360
gtctgagtag gtgtcattct attctggggg gtggggtggg gcaggacagc aagggggagg 3420
attgggaaga caatagcagg catgctgggg atgcggtggg ctctatggct tctgaggcgg 3480
aaagaaccag ctggggctct agggggtatc cccacgcgcc ctgtagcggc gcattaagcg 3540
cggcgggtgt ggtggttacg cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg 3600
ctcctttcgc tttcttccct tcctttctcg ccacgttcgc cggctttccc cgtcaagctc 3660
taaatcgggg catcccttta gggttccgat ttagtgcttt acggcacctc gaccccaaaa 3720
aacttgatta gggtgatggt tcacgtagtg ggccatcgcc ctgatagacg gtttttcgcc 3780
ctttgacgtt ggagtccacg ttctttaata gtggactctt gttccaaact ggaacaacac 3840
tcaaccctat ctcggtctat tcttttgatt tataagggat tttggggatt tcggcctatt 3900
ggttaaaaaa tgagctgatt taacaaaaat ttaacgcgaa ttaattctgt ggaatgtgtg 3960
tcagttaggg tgtggaaagt ccccaggctc cccaggcagg cagaagtatg caaagcatgc 4020
atctcaatta gtcagcaacc aggtgtggaa agtccccagg ctccccagca ggcagaagta 4080
tgcaaagcat gcatctcaat tagtcagcaa ccatagtccc gcccctaact ccgcccatcc 4140
cgcccctaac tccgcccagt tccgcccatt ctccgcccca tggctgacta attttttta 4200
tttatgcaga ggccgaggcc gcctctgcct ctgagctatt ccagaagtag tgaggaggct 4260
tttttggagg cctaggcttt tgcaaaaagc tcccgggagc ttgtatatcc attttcggat 4320
ctgatcagca cgtgttgaca attaatcatc ggcatagtat atcggcatag tataatacga 4380
caaggtgagg aactaaacca tggccaagtt gaccagtgcc gttccggtgc tcaccgcgcg 4440
cgacgtcgcc ggagcggtcg agttctggac cgaccggctc gggttctccc gggacttcgt 4500
ggaggacgac ttcgccggtg tggtccggga cgacgtgacc ctgttcatca gcgcggtcca 4560
ggaccaggtg gtgccggaca acaccctggc ctgggtgtgg gtgcgcggcc tggacgagct 4620
gtacgccgag tggtcggagg tcgtgtccac gaacttccgg gacgcctccg ggccggccat 4680
gaccgagatc ggcgagcagc cgtgggggcg ggagttcgcc ctgcgcgacc cggccggcaa 4740
ctgcgtgcac ttcgtggccg aggagcagga ctgacacgtg ctacgagatt tcgattccac 4800
cgccgccttc tatgaaaggt tgggcttcgg aatcgttttc cgggacgccg gctggatgat 4860
cctccagcgc ggggatctca tgctggagtt cttcgcccac cccaacttgt ttattgcagc 4920
ttataatggt tacaaataaa gcaatagcat cacaaatttc acaaataaag catttttttc 4980
actgcattct agttgtggtt tgtccaaact catcaatgta tcttatcatg tctgtatacc 5040
gtcgacctct agctagagct tggcgtaatc atggtcatag ctgtttcctg tgtgaaattg 5100
ttatccgctc acaattccac acaacatacg agccggaagc ataaagtgta aagcctgggg 5160
tgcctaatga gtgagctaac tcacattaat tgcgttgcgc tcactgcccg ctttccagtc 5220
gggaaacctg tcgtgccagc tgcattaatg aatcggccaa cgcgcgggga gaggcggttt 5280
gcgtattggg cgctcttccg cttcctcgct cactgactcg ctgcgctcgg tcgttcggct 5340
gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag aatcagggga 5400
taacgcagga aagaacatgt gagcaaaagg ccagcaaaag gccaggaacc gtaaaaaggc 5460
cgcgttgctg gcgtttttcc ataggctccg cccccctgac gagcatcaca aaaatcgacg 5520
ctcaagtcag aggtggcgaa acccgacagg actataaaga taccaggcgt ttccccctgg 5580
aagctccctc gtgcgctctc ctgttccgac cctgccgctt accggatacc tgtccgcctt 5640
tctcccttcg ggaagcgtgg cgctttctca atgctcacgc tgtaggtatc tcagttcggt 5700
gtaggtcgtt cgctccaagc tgggctgtgt gcacgaaccc cccgttcagc ccgaccgctg 5760
cgccttatcc ggtaactatc gtcttgagtc caacccggta agacacgact tatcgccact 5820
ggcagcagcc actggtaaca ggattagcag agcgaggtat gtaggcggtg ctacagagtt 5880
cttgaagtgg tggcctaact acggctacac tagaaggaca gtatttggta tctgcgctct 5940
gctgaagcca gttaccttcg gaaaaagagt tggtagctct tgatccggca aacaaaccac 6000
cgctggtagc ggtggttttt ttgtttgcaa gcagcagatt acgcgcagaa aaaaaggatc 6060
tcaagaagat cctttgatct tttctacggg gtctgacgct cagtggaacg aaaactcacg 6120
ttaagggatt ttggtcatga gattatcaaa aaggatcttc acctagatcc ttttaaatta 6180
```

```
aaaatgaagt tttaaatcaa tctaaagtat atatgagtaa acttggtctg acagttacca 6240
atgcttaatc agtgaggcac ctatctcagc gatctgtcta tttcgttcat ccatagttgc 6300
ctgactcccc gtcgtgtaga taactacgat acgggagggc ttaccatctg gccccagtgc 6360
tgcaatgata ccgcgagacc cacgctcacc ggctccagat ttatcagcaa taaaccagcc 6420
agccggaagg gccgagcgca gaagtggtcc tgcaacttta tccgcctcca tccagtctat 6480
taattgttgc cgggaagcta gagtaagtag ttcgccagtt aatagtttgc gcaacgttgt 6540
tgccattgct acaggcatcg tggtgtcacg ctcgtcgttt ggtatggctt cattcagctc 6600
cggttcccaa cgatcaaggc gagttacatg atcccccatg ttgtgcaaaa aagcggttag 6660
ctccttcggt cctccgatcg ttgtcagaag taagttggcc gcagtgttat cactcatggt 6720
tatggcagca ctgcataatt ctcttactgt catgccatcc gtaagatgct tttctgtgac 6780
tggtgagtac tcaaccaagt cattctgaga atagtgtatg cggcgaccga gttgctcttg 6840
cccggcgtca atacgggata ataccgcgcc acatagcaga actttaaaag tgctcatcat 6900
tggaaaacgt tcttcggggc gaaaactctc aaggatctta ccgctgttga gatccagttc 6960
gatgtaaccc actcgtgcac ccaactgatc ttcagcatct tttactttca ccagcgtttc 7020
tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg cgacacggaa 7080
atgttgaata ctcatactct tcctttttca atattattga agcatttatc agggttattg 7140
tctcatgagc ggatacatat ttgaatgtat ttagaaaaat aaacaaatag gggttccgcg 7200
cacatttccc cgaaaagtgc cacctgacgt c                                7231
```

<210> 43
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 43
tgtcttggat ccaagatgaa gcgcgcccgc cccagcgaag atgacttc 48

<210> 44
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 44
aaacacggcg gccgctcttt cattcttg 28

<210> 45
<211> 17
<212> PRT
<213> Ad 37 N-terminus

<400> 45
```
Met Ser Lys Arg Leu Arb Val Glu Asp Asp Phe Asn Pro Val Tyr Pro Tyr
 1               5                  10                  15
```

<210> 46
<211> 6
<212> PRT
<213> artificial sequence

<400> 46
```
Lys Arg Ala Arg Pro Ser
 1               5
```

<210> 47
<211> 17

<212> PRT
<213> Ad5 modified N-terminus

<400> 47
Met Lys Arg Ala Arg Pro Ser Glu Asp Thr Phe Asn Pro Val Tyr Pro Tyr
1 5 10 15

<210> 48
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 48
ggatccatgg gatacttggt agca 24

<210> 49
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 49
gcaactcgag tcattcttgg gcaatatagg 30

<210> 50
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 50
cgcgctgact cttaaggact agtttc 26

<210> 51
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 51
gcgcttaatt aacatcatca ataatatacc ttatttt 37